

LabBook

4 July 2016:

- ✓ Bottles of LB and LB-Agar (200 mL and 400 mL):
LB-Agar: 14 g for 400 mL of water
LB: 8 g for 400 mL of water
Autoclave
- ✓ Resistance test of *P. putida*:
2 plates of LB-Agar with either Kanamycin (50 µg/mL) or Spectinomycin (50 µg/mL).
- ✓ Preparation of HEPES solution 0.5 M (for electroporation):
11 g of HEPES (solid) in 64 mL of water
NaOH (0.1 M) for pH7
- ✓ Refresh *P. putida* (the Petri dish got old)
- ✓ *E. coli* C118 with plasmids pSEVA2311 (KanR) and pSEVA224 (KanR) were separately spread in LB.
 - cells with pSEVA2311 were observed
 - cells with pSEVA224 were not observed!! → Jonathan asked for another sample

5 July 2016:

- ✓ LB culture of *E. coli* C118
- ✓ HEPES filtration
- ✓ Check the inventory of the fridge
 - Plate of *P. putida* at 30 °C and 37 °C → We didn't do this step
- ✓ Liquide culture (5 mL of preculture) of *P. putida* to have competent cells

6 July 2016:

- ✓ Preculture for transformation (5 mL) of *E. coli* DH5 alpha

7 July 2016:

- ✓ Miniprep of *E. coli* C118 with pSEVA2311
Protocol "Midiprep" with 50 mL of culture

C = 80 ng/ μ L in 200 μ L of elution buffer

OD₂₆₀ = 1.616

OD₂₈₀ = 0.8

- ✓ Transformation of *Pseudomonas* with pSEVA2311
40 mL of culture (competent cell):
When OD= 0.6:
 - 20 mL for Heat Shock
 - 20 mL for Electroporation
- ✓ After electroporation and heat-shock spread on Amp-Plates

8 July 2016:

- ✓ The transformation of 07/07/2016 didn't work because the plasmid given by Robert didn't match with *Pseudomonas* → Second transformation (Heat shock and electroporation)
- ✓ Preparation of plates (LB+Antibiotics):
 - 10 plates LB-Agar, Kanamycin (50 μ g/mL)
 - 10 plates LB-Agar, Chloramphenicol (25 μ g/mL)
 - 10 plates LB-Agar, Ampicillin (100 μ g/mL)
 - 10 plates LB-Agar, without antibiotic

12 July 2016:

- ✓ Preparation of M9-media (10X, V= 600 mL) with different kind of C-sources:
 - Glucose → DONE
 - Glycerol → DONE
 - Fructose → NO YET
 - Benzoate → NO YET
 - Lactic acid → NO YET
 - Methane → NO YET

Compounds	Cf	Mass (g) or volumes (mL)
CaCl ₂ .H ₂ O	1 mM	0.0882 g
MgSO ₄	20 mM	1.44 g
FeSO ₄ .H ₂ O	0.1 mM	0.01668 g
M9 (salt)	10 X	67.68 g
Glycerol (100 %) *	8 % v/v	60.48 g
Casamino acid	2 % w/v	12 g
Thiamine 10 mg/mL **	10 μ g/mL	(0.6 g)
Uracil	200 μ g/mL	0.12 g

Leucine	300 µg/mL	0.18 g
NaOH	pH 6.6	-

*Glucose: 59.22 g;

** Thiamine has not been added but it should be before the preculture of *P. putida*.

→ problem: precipitation

13 July 2016

- ✓ Preparation of the deletion process – Transformation of *E. coli* Mach1 competent cells (CaCl₂ treatment) by Heat Shock:

- Plasmids pKD46, pCP20 and pKD4
- 3 tubes where 25 µL of cells were mixed with 1.5 µL of each plasmid (pKD4, pCP20 or pKD46)
- Incubation on ice 20 min
- Heat at 42 °C (thermocycler) 1 min
- 120 µL of LB added to each tube
- Incubation: 30 min at 37 °C for the plasmid pKD4, 30 °C for the plasmids pCP20 and pKD46
- Spread on plates (Ampicillin 100 µg/mL)
- Incubation overnight at 37 °C for pKD4, 30 °C for pCP20 and pKD46

- ✓ Preparation for the deletion process – Preparation of *E. coli* DH5 alpha competent cells:

- 5 mL of preculture of DH5 alpha were prepared
- Incubation overnight at 37 °C

14 July 2016:

- ✓ pKD46 and pCP20 transformation properly worked
- ✓ pKD4 didn't succeed
- ✓ Preparation of 5 mL culture of pKD46 and pCP20 to purify plasmid
- ✓ pKD4 since it is not needed for transformation will be used w/o amplification
- ✓ DH5 alpha competent cells were prepared and stored

15 July 2016:

- ✓ Midiprep of pCP20 and pKD46:
 - pCP20: 65 ng/µL
 - pKD46: 50 ng/µL

- ✓ Transformation of *P. putida* KT2440 with pKD46 plasmid to start deletion process
Incubation overnight at 30 °C (Amp Plates)
- ✓ Spread KT2440 WT and check growth at 37 °C

16 July 2016:

- ✓ Check transformation results → cells grew on Ampicillin at 30 °C
- ✓ Pick 4 clones from previous plate and spread them in a new plate (Amp with proper concentration) and incubate it at 30 °C.

Pseudomonas Putida: Working concentration of Antibiotics

Kan (50 µg/mL) → 1.7 µL of stock solution/mL

Amp (500 µg/mL) → 5 µL of stock solution/mL

Spec (50 µg/mL) → 1 µL of stock solution/mL

- ✓ KT2440 grew well at 37 °C

18 July 2016:

- ✓ Electroporation of *P. putida*: pKD46
 - Electro-competent cells have been prepared
 - 1.5 µL of DNA were added to ~ 50 µL of electro-competent cells (all the tube)
 - 1 mL of LB was transfer in a 1.5 mL tube
 - 500 µL of the LB were prepare in the pipette-tip and put rapidly in the electro-tank after electroporation (at 1800 V)
 - All the cells + LB of the tank were transferred to the 1.5 mL tube containing LB
 - Incubation + Shaking 1 h at 30 °C
 - Centrifugation 3 min 90 rpm
 - Cells were plated in LB Agar Ampicillin (100 µg/µL) and incubated at 30 °C
- ✓ Spread C118 (pSEVA224) on plates with Kanamycin

19 July 2016:

- ✓ The transformation (Electroporation of 18/08) didn't work → maybe the replicon doesn't match with *P. putida*
- ✓ Colony PCR: to check the transformation of *P. putida* with p2311
 1. Tick one colony and with the same tip, spread a little area on LB plate and dissolve the rest in 10 µL ddH₂O
 2. Gently vortex and briefly centrifuge DreamTaq Green PCR Master Mix (2X) after thawing.
 3. Place a thin-walled PCR tube on ice and add the following components for each 50 µL reaction:

DreamTaq Green PCR Master Mix (2X)	1X	25 µL
Forward primer	0.5 µM	0.25 µL
Reverse primer	0.5 µM	0.25 µL
Colony water	----	2 µL
Water, nuclease-free	----	22.5 µL

4. Gently vortex the samples and spin down.
5. Perform PCR using the recommended heat cycling conditions outlined below:

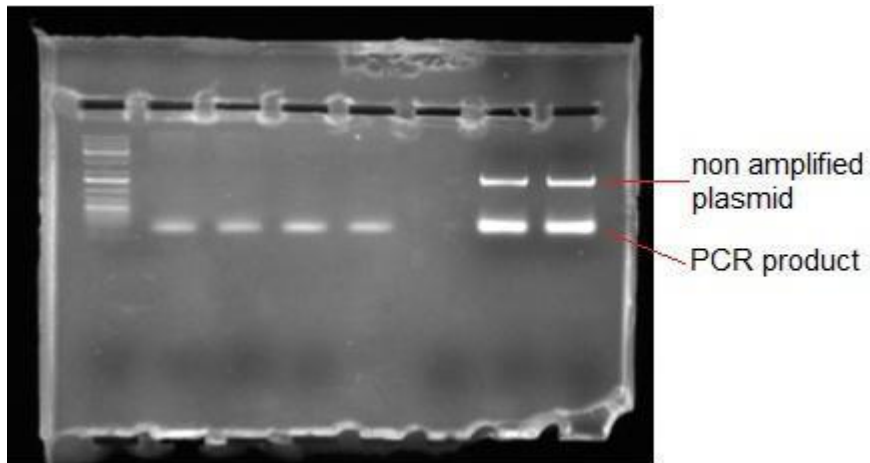
Step	Temperature °C	Time	Number of cycles
Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	30 s	30
Annealing	55(T _m)-5 °C	30	
Extension	72 °C	1 min/kb	
Final extension	72 °C	10 min	1
Pause	4 °C	“00”	---

6. Load 5-15 µL of PCR mixture directly on a gel.

- ✓ Electrophoresis to check the transformation

- 1 % of agarose
- Replicates have been done

PCR Colony					PCR		
Marker	Pseudo. HeatShock 1	Pseudo. HeatShock 2	Pseudo. Electropor. 1	Pseudo. Electropor. 2	—	PCR product 1	PCR product 2



- ✓ PCR of pKD4:
- Mix of 100 μ L

Reaction buffer	20 μ L
dNTP mix	2 μ L
Primers (reverse and forward)	0.5 μ L
Template (pKD4)	1 μ L
Q5 polymerase	1 μ L
H2O	Qs 100 μ L (75.5 μ L)

- Cycles

Temperature	Time	Number of cycles
98 °C	30''	1
90 °C	30''	32
60 °C	30''	
72 °C	1'	
72 °C	5'	1
4 °C	“00”	---

✓ Culture of *E. coli* C118 pSEVA for midi prep'

20 July 2016:

✓ Midiprep' of pSEVA224 (from *E. coli*)
C= 59.9 ng/μL in 200 μL of elution buffer

$$1. \text{OD}_{260}/\text{OD}_{280} = 1.97$$

✓ Alternative to delete gene:

Transformation of *Pseudomonas* by a PCR product: Kanamycin resistance cassette + homologous region 5' and 3' using Heat Shock and Electroporation.

→ See protocols for both transformations

→ We used 10 μL of DNA

✓ Preparation of 7 plates for *Pseudomonas* (1.7 μL/mL of kanamycin stock solution)

✓ Liquid culture of *E. coli* pSEVA224 (2 mL):

- 2 mL of LB
- 3.4 μL of Kan → We made a mistake: we should have put 2 μL of Kan (since we used *E. coli*)

- ✓ Preculture to amplify the vector given by Cyril (BBa.R0010 pLac)

- *E. coli* G3A
- 50 mL of LB
- 50 µL of Amp

21 July 2016:

!!! The fridge and freezer were off.

- ✓ The transformation of the PCR product (for the deletion process) didn't work
 2. We will focus on the construction of the plasmid
- ✓ Store the culture (in glycerol (1 vol of glycerol+1 vol of the culture))
- ✓ Midiprep of the vector R0010
 3. 325 ng/µL
 4. $OD_{260}/OD_{280} = 1.8$
- ✓ Preparation of Ampicillin solution stock 50 mg/mL in H₂O (Helix) aliquoted in 5 tubes of 2 mL.

In sterile condition:
 0.5 g of Amp + 10 mL of H₂O
 Filtration (0.2 µm)
 Storage away from light
- ✓ Digestion of:
 - the vector (with Ba.R0010 pLac provided by Cyril) by EcoRI (E) and SpeI (S)
 - the vector (with Ba.R0010 pLac provided by Cyril) by EcoRI and PstI (P)
 - the phaC sequence by EcoRI and SpeI
 - the Propionyl-CoA synthase sequence by EcoRI and PstI
- By the way, we tried to remove the promoter (BBa.R0010 pLac) from the vector.
- 100 µL of water were added to the phaC sequence and the Propionyl-CoA synthase sequence.

	Vector (E/S)	phaC (E/S)	Vector (E/P)	propCoA(E/P)
DNA	2 µL	10 µL	2 µL	10 µL
Buffer	Cutsmart 10X: 3 µL	Cutsmart 10X: 3 µL	2.1: 3 µL	2.1: 3 µL

Enzymes	EcoRI: 1 μ L SpeI: 1 μ L	EcoRI: 1 μ L SpeI: 1 μ L	EcoRI: 1 μ L PstI: 1.5 μ L	EcoRI: 1 μ L PstI: 1.5 μ L
H2O	23 μ L	15 μ L	22.5 μ L	14.5 μ L

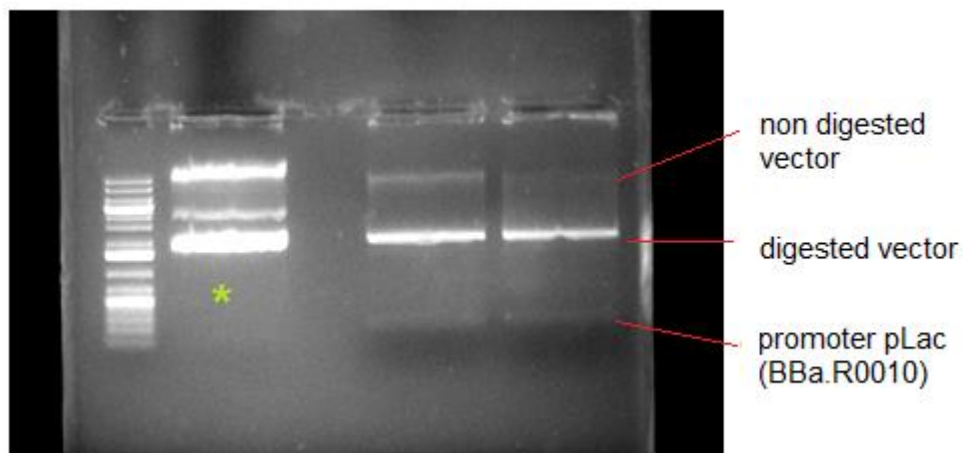
Incubation 1 h at 37 °C

✓ Electrophoresis

- 1 % of Agarose

Wells:	Marker (8 μL)	Control vector with pLac (5 μL DNA+1 μL loading buffer)	Vector E/S (30 μL DNA +6 μL loading buffer)	Vector E/S (30 μL DNA +6 μL loading buffer)
Expected results	—	1 band (2270 bp)	2 bands (2070 bp + 200 bp)	2 bands (2070 bp + 200 bp)

- Vector (with pLac): 2270 bp
- Promoter pLac ~ 200 bp
- Results



*circular plasmid (3 forms: compacted, intermediate and loose forms)

- We get rid of the promoter and we kept the empty vectors

✓ Purification of the DNA sequences phaC and prop-CoA synth

- Zymoclean Gel DNA Recovery Kit
- Elution with 6 μ L of water

- Wait 1-2 min before the last spin
- The check at the spectrophotometer nanodrop showed a pic around 240nm
 Maybe: problem with the wash buffer reminded in the column
 Inversion of the 2 columns before the second spin

22 July 2016:

✓ Ligations

Empty vector (R0010)	10 µL
Insert phaC	20 µL
T4 buffer 10X	4 µL
T4 ligase	2 µL
H2O	5 µL

Empty vector (R0010)	10 µL
Insert propCoA synth.	20 µL
T4 buffer 10X	4 µL
T4 ligase	2 µL
H2O	5 µL

- Incubation 1 h at RT

✓ Transformation of *E. coli* DH5 alpha, Top 10 and BL21 by Heat Shock

- Same protocol
- We didn't recover enough DNA, insert from previous midiprep, purification.
- These strains were spread on labeling plates:

22/07/16 <i>E. coli</i> phaC DH5 alpha	22/07/16 <i>E. coli</i> phaC Top10	22/07/16 <i>E. coli</i> phaC BL21
22/07/16 <i>E. coli</i> propCoA DH5 alpha	22/07/16 <i>E. coli</i> propCoA Top10	22/07/16 <i>E. coli</i> propCoA BL21

- Incubation overnight at 37 °C

23 July 2016:

- ✓ “Transformed” clones were observed.
- ✓ Colony PCR.

25 July 2016:

✓ Media:

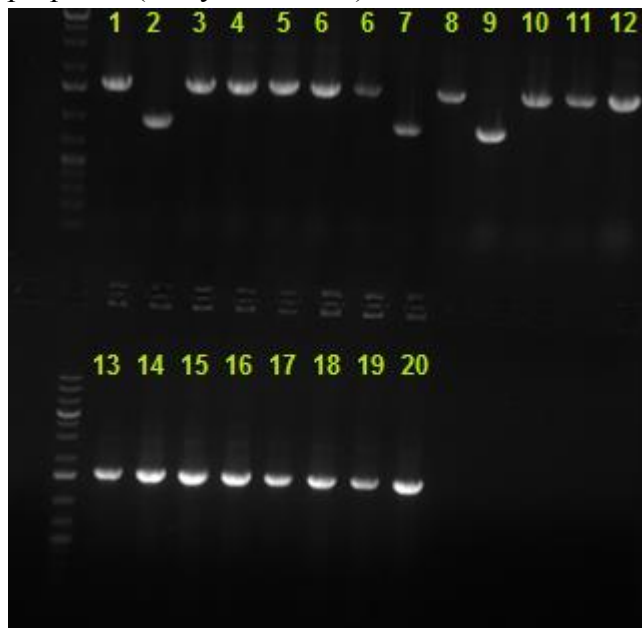
- 5 bottle of LB:
 - 3 x 400 mL
 - 2 x 200 mL
- 5 bottle of LB-Agar:
 - 3 x 400 mL
 - 2 x 200 mL

✓ Previous transformations (22/07) worked.

✓ Electrophoresis (PCR colony 23/07)

Gel 1 %

- propCoA (in Cyril's vector):



phaC (in Cyril's vector):



✓ Precultures:

- 2 mL of LB
- Amp (1 μ L/mL of stock solution)
- A good colony:
 - phaC: 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 19, 20.
 - propCoA: 1, 3, 4, 5, 6, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20.
- Incubation overnight at 37 °C

26 July 2016:

✓ Miniprep' kit Sigma-Aldrich: extraction of the plasmids from the precultures of the 26th of July

- For the propCoA transferase: problem with the 8th tube we lost a lot of bacteria*

Colonies (with the phaC)	1	2	4	5	6	7	8	9	10
Concentrations of DNA (ng/ μ L)	282.3	170.4	174.6	176.5	188.9	167.8	185.9	191.4	173.4
Colonies (with the phaC)	11	12	13	14	15	16	17	19	20
Concentrations of DNA (ng/ μ L)	160.9	173.0	170	185.3	162	189.5	193.6	183	163

Colonies (with the propCoA)	1	3	4	5	6	8	10	11	12
Concentrations of DNA (ng/ μ L)	150.5	143.4	156	151.8	234.2	29.4*	194.5	167.2	162.8
Colonies (with the propCoA)	13	14	15	16	17	18	19	20	-
Concentrations of DNA (ng/ μ L)	169.4	282.8	192.3	226	203.3	175.1	256.5	181.7	-

- ✓ M9 medium 2 x 50 mL of 2.5 X
 - Without C-sources, thiamine.
 → **Problem: precipitation (the same than previous M9 media)**

27 July 2016:

- ✓ Stock solutions:
 - CaCl₂ 1 M: 27.745 g in 250 mL of milliQ
 - MgSO₄ 1 M: 30.1 g in 250 mL of milliQ
 - FeSO₄ 10 mM: 0.379 g in 250 mL of milliQ
- ✓ Preculture of *Pseudomonas putida* KT2440 (2 mL)

27 July 2016:

- ✓ Aliquots for sequencing V= 20 μ L
- ✓ Medium M9 1X without Carbone source and filtered (2 μ m) in 2 falcon tubes of 50 mL:
 - Casamino acid: 1.2 g
 - Leucine: 3 mg
 - Uracil: 2 mg
 - Thiamine 10 mg/mL: 10 μ L
 - M9 salt 5X: 20 mL
 - CaCl₂ 1 M: 10 μ L
 - MgSO₄ 1 M: 100 μ L
 - FeSO₄ 10 mM: 1 μ L
 - Water milliQ qs 100 mL
 - NaOH for pH between 6 and 7.4 → no need since the pH was already at 6.8**No precipitation!**

✓ 10 mL of Media with C-source:

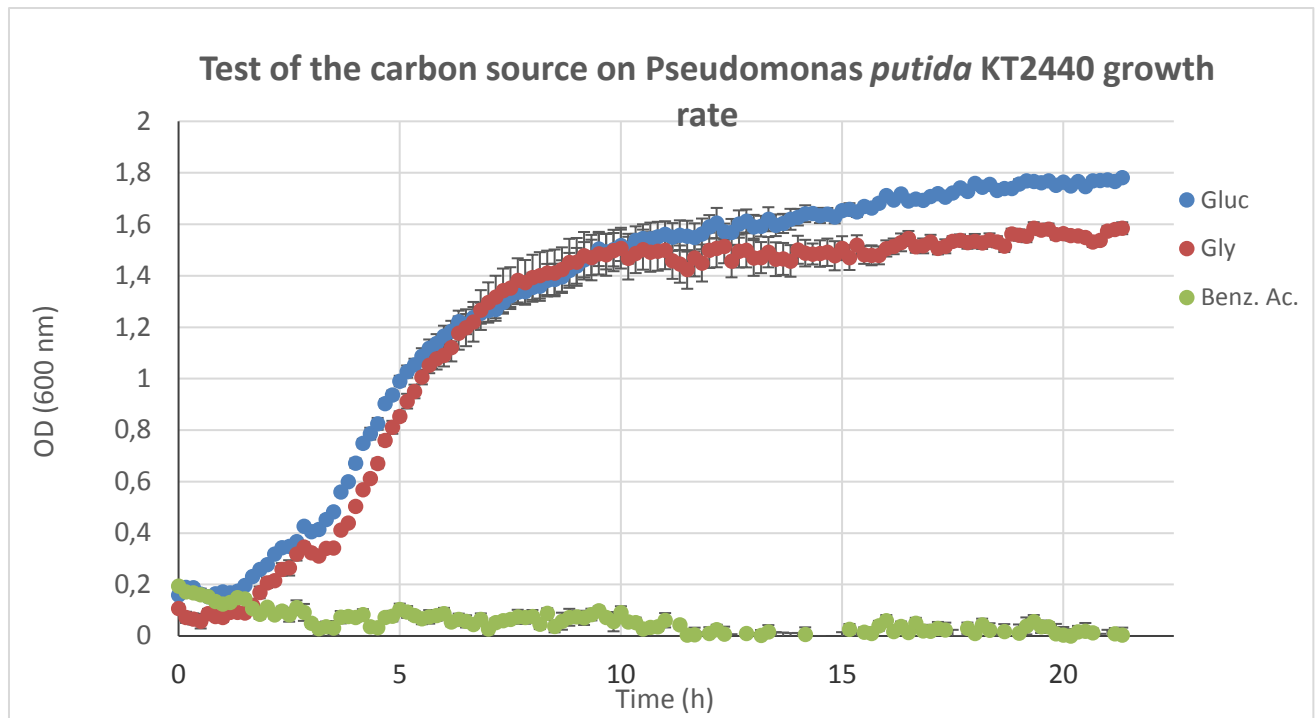
- 0.10037 g of Glycerol (100 %) (*0.16 put*) in 10 mL of milliQ water
- 0.0982 g of Glucose in 10 mL of milliQ water
- 0.057 g of Benzoic acid in 10 mL of milliQ water

✓ Preparation for plate (96 wells) and Growth rate with the CLARIO star machine:

- 26.7 µL of the overnight preculture of *Pseudomonas* in medium M9 (with either Benzoic acid, Glucose or Glycerol) qs 1 mL to have around OD= 0.005.

	1	2	3	4
A	-	Benzoic acid M9 medium	Glucose M9 medium	Glycerol M9 medium
B	Benzoic acid M9 medium	BE-M9 with cells	BE-M9 with cells	BE-M9 with cells
C	Glucose M9 medium	GLU-M9 with cells	GLU-M9 with cells	GLU-M9 with cells
D	Glycerol M9 medium	GLY-M9 with cells	GLY-M9 with cells	GLY-M9 with cells

- Plate mode, slow kinetic, OD_{600 nm}, T= 30 °C, shaking (200 rpm) before cycle of 10 min



Growth rate of *Pseudomonas putida* in M9 minimal medium supplemented with 3 different carbon source.

2 August 2016

Resolubilization of IDT gBlocks in 100 μL water ($C_f = 10 \text{ ng}/\mu\text{L}$)

Digestion

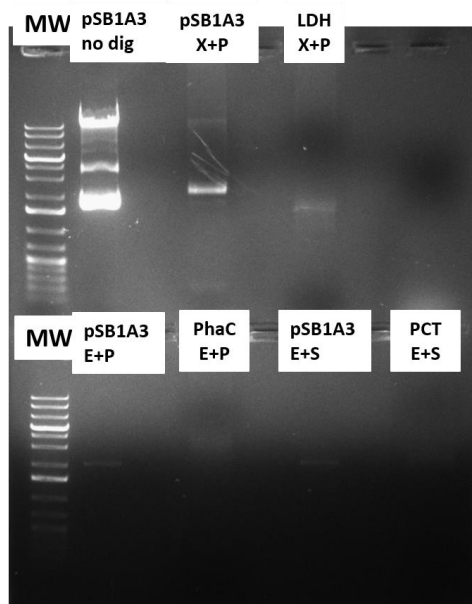
Mix:

	PhaC2	PCT1	LDH1	pSB1A3
GBlock gene or plasmid	10 μL	10 μL	10 μL	2 μL ($\approx 600 \text{ ng}$)
Buffer NEB	3 μL (2.1)	3 μL (CutSmart)	3 μL (2.1)	3 μL (CutSmart or 2.1)*
Enzyme 1	1 μL (EcoRI)	1 μL (EcoRI)	1 μL (XbaI)	1 μL (EcoRI or XbaI)
Enzyme 2	1 μL (PstI)	1 μL (SpeI)	1 μL (PstI)	1 μL (PstI or SpeI)
H ₂ O (qs 30 μL)	15 μL	15 μL	15 μL	23 μL

*CutSmart when we don't digest by PstI and 2.1 when PstI is used

Incubation 1 h 37 $^{\circ}\text{C}$

Agarose gel 1 %



→ Not enough gene on the gel to detect it!

Digestion

Mix:

	PhaC2	PCT1	LDH1
GBlock gene	10 μ L	10 μ L	10 μ L
Buffer NEB	3 μ L (2.1)	3 μ L (CutSmart)	3 μ L (2.1)
Enzyme 1	1 μ L (EcoRI)	1 μ L (EcoRI)	1 μ L (XbaI)**
Enzyme 2	1 μ L (PstI)	1 μ L (SpeI)*	1 μ L (PstI)
H2O (qs 30 μ L)	15 μ L	15 μ L	15 μ L

* PCT1 has a PstI restriction site in its sequence so the digestion was done by SpeI

** LDH1 has a EcoRI restriction site in its sequence so the digestion was done by XbaI

Incubation 1 h 37 °C

DNA clean-up → kit Monarch PCR and DNA clean-up (#T1030G) NEB

DNA extraction from gel

→ DNA Gel extraction kit (#T1020G) NEB

Incubation at 55 °C to dissolve gel

400 μ L buffer for 100 mg of agarose gel

DNA elution in 40 μ L water

Ligation

10 µL Vector (pSB1A3 digested)
20 µL Insert digested
4 µL 10X T4 DNA ligase buffer
2 µL T4 DNA ligase
5 µL H₂O
Incubation RT 1 h

Transformation

- Thaw 25 µL of competent DH5α *E. coli*
- Add 10 µL of ligated DNA
- 20 min incubation in ice
- 1 min 42 °C
- Add 120 µL of LB medium
- Incubation 40 min 37 °C
- Spread on plates LBA (Ampicillin)
- Incubation 37 °C overnight

3 August 2016

Colonies PCR

Mix (50 µL total volume reaction):

- 25 µL DreamTaq PCR Mastermix 2X (ThermoScientific)
- 5 µL 10X DreamTaq Green Buffer (ThermoScientific)
- 0.25 µL Forward Primer (0.5 µM) iG001
- 0.25 µL Reverse Primer (0.5 µM) iG002
- 19.5 µL H₂O filtrated (qs 50 µL)

Mix prepared for 15 reactions (plus one reaction prepared without Green buffer)
+ clones

PCR program:

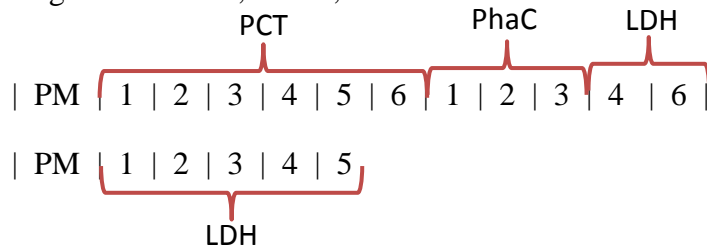
- 95 °C 5 min
 - 95 °C 30 s
 - 50 °C 30 s
 - 72 °C 2 min
 - 72 °C 10 min
 - 10 °C ∞
- } ×30

Nanodrop

PhaC2 3.5 ng/µL
PCT1 3.8 ng/µL
LDH1 8.7 ng/µL
pSB1A3 (digested by X+P) 3.3 ng/µL

Gel electrophoresis

Migration 30 min, 100 V, 300 Ma



⇒ The results were not conclusive

4 August 2016

Colonies PCR

Mix (25 µL total volume reaction):

- 12.5 µL DreamTaq PCR Mastermix 2X (ThermoScientific)
- 2.5 µL 10X DreamTaq Green Buffer (ThermoScientific)
- 0.25 µL Forward Primer (0.5 µM) iG001
- 0.25 µL Reverse Primer (0.5 µM) iG002
- 9.5 µL H₂O filtrated (qs 25 µL)

Mix prepared for 7 reactions

+ clones

PCR program:

- 95 °C 5 min
 - 95 °C 30 s
 - 50 °C 30 s
 - 72 °C 2 min
 - 72 °C 10 min
 - 10 °C ∞
- ×30

⇒ The results were not conclusive

8 August 2016

Ligation with:

- 50 ng vector
- 5 µL T4 DNA ligase Buffer 10X
- 3 µL T4 DNA
- H₂O qs for 50 µL
- DNA

PhaC 2 (3.5 ng/ μ L)	11.7 μ L	Vector E/P (6.4 ng/ μ L)	7.8 μ L	H2O 22.5 μ L
PCT 1 (3.8 ng/ μ L)	30.4 μ L	Vector E/S (12.2 ng/ μ L)	4.1 μ L	H2O 7.5 μ L
LDH 1 (8.7 ng/ μ L)	13.9 μ L	Vector X/P (5.9 ng/ μ L)	8.5 μ L	H2O 19.6 μ L

We put 27.5 μ L of Pct1 DNA because there was not enough.

Transformation:

Thaw DH5-alpha competent bacteria in the ice.
Using the protocol processing by heat shock.
25 μ L of bacteria + Mix of ligation (2 μ L each time)
Incubation 20 minutes in ice and then 1 min at 42 ° C
Adding 120 μ L LB and incubate at 37 ° for 1 hour
Spread on LB Ampicillin.
Incubation overnight 37 °C.

9 August 2016

Colonies PCR

Mix PCR with 25 μ L of total volume reaction prepared for 12 reactions prepared as before (DreamTaq #K1071 Thermo Scientific).

PCR program:

- 95 °C 5 min
 - 95 °C 30 s
 - 50 °C 30 s
 - 72 °C 1 min
 - 72 °C 10 min
 - 10 °C ∞
- } $\times 30$

\Rightarrow No bands on the gel (maybe problems with the enzyme)

10 August 2016

Digestion

Mix:

	PhaC2	PhaC3	PCT2	LDH1
GBlock gene	20 μ L	20 μ L	20 μ L	20 μ L
Buffer NEB	3 μ L (2.1)	3 μ L (2.1)	3 μ L (CutSmart)	3 μ L (2.1)
Enzyme 1	1 μ L (EcoRI)	1 μ L (EcoRI)	1 μ L (EcoRI)	1 μ L (XbaI)*

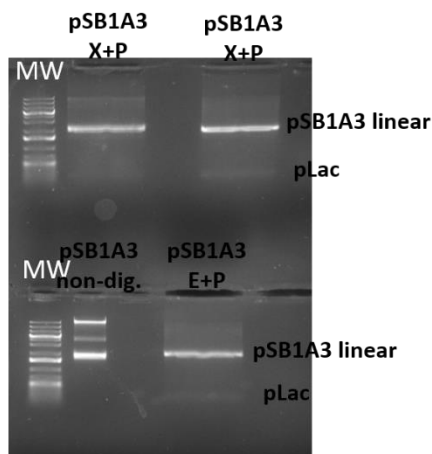
Enzyme 2	1 μ L (PstI)	1 μ L (PstI)	1 μ L (PstI)	1 μ L (PstI)
H2O (qs 30 μ L)	5 μ L	5 μ L	5 μ L	5 μ L

* LDH1 has a EcoRI restriction site in its sequence so the digestion was done by XbaI

	pSB1A3	pSB1A3
Vector DNA	2 μ L (\approx 600 ng)	6 μ L (\approx 2 μ g)
Buffer NEB	3 μ L (CutSmart or 2.1)*	9 μ L (CutSmart or 2.1)*
Enzyme 1	1 μ L (EcoRI)	3 μ L (XbaI)
Enzyme 2	1 μ L (PstI)	3 μ L (PstI)
H2O (qs 30 μ L)	23 μ L	69 μ L
		Divided in 3 reaction tubes

Incubation 1 h 37 °C

1 % agarose gel



▲ 2 trash nucleotides after the PstI restriction site in the suffix were forgotten so the digestion risks to fail →

▲ PhaC genes doesn't match the gene in our reference publications but it corresponds to the gene used by the YALE IGEM team in 2013

11 August 2016

Extraction of digested vectors (pSB1A3 X+P and pSB1A3 E+P) from agarose gel (kit Zymoclean gel DNA Recovery kit #D4001S)

Concentration of the samples:

pSB1A3 digested by X+P → 3.9 ng/μL
pSB1A3 digested by E+P → 7.2 ng/μL

Digestion by SpeI

Mix:

- 20 μL genes digested by E+P or X+P or vector digested by X+P
- 3 μL buffer NEB CutSmart
- 1 μL enzyme SpeI
- 6 μL H2O (qs 30 μL)

Or mix:

- 50 μL genes digested by E+P or X+P or vector digested by X+P
- 6 μL buffer NEB CutSmart
- 2 μL enzyme SpeI
- 2 μL H2O (qs 60 μL)

DNA purification

DNA clean & concentrator-5 kit (#D4003S Zymo Research)

For the vector: 2 volumes of binding buffer add to DNA (ratio 2:1)

For the genes: 5 volumes of binding buffer add to DNA (ratio 5:1)

Elution with 20 μL of water and incubation 2 min before centrifugation for DNA elution

Ligation

Mix:

	PhaC2	PhaC3	PCT2
Vector pSB1A3	4.5 μL	4.5 μL	4.5 μL
Gene	14 μL	11 μL	10 μL
T4 DNA ligase Buffer	3 μL	3 μL	3 μL
T4 DNA ligase	1 μL	1 μL	1 μL
H2O (qs 30 μL)	7.5 μL	10.5 μL	11.5 μL

Incubation 1 h RT

Sample frozen at -20 °C until transformation

12 August 2016

Transformation

Thaw DH5-alpha competent bacteria in the ice.

Using the protocol processing by heat shock.

25 μL of bacteria + 5 μL ligation mix

Incubation 20 minutes in ice

1 min at 42 ° C

3 min in ice

Adding 120μl of LB

Incubation at 37 ° for around 50 min
Spread on LB Ampicillin plates
Incubation overnight 37 °C

13 August 2016

Results transformation:

We got clones from the transformation. We took 3 clones from Pct(v2), 2 from PhaC(v2) and 2 from PhaC(v3) and performed PCR colony.

PCR Colonies:

20 µL mix per sample with:

- 10 µL Green Taq Master Mix 2X (#K1081 Thermo Scientific)
- 0.25 µL Forward primer iG001 (10 µM)
- 0.25 µL Forward primer iG002 (10 µM)
- 9.5 µL H₂O (qs 20 µL)

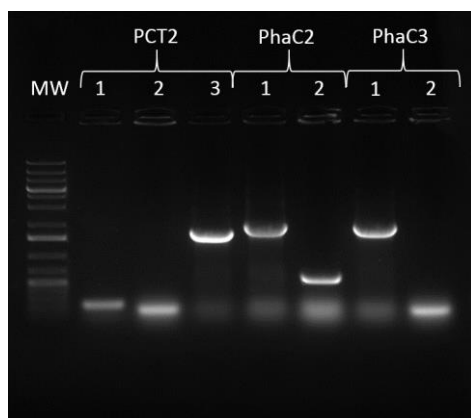
Mix for 9 reactions

+ clones

PCR program:

- 1) 95 °C 5 min
 - 2) 95 °C 30 s
 - 3) 50 °C 30 s
 - 4) 72 °C 2 min
- 30 repeats of the steps bloc from 2) to 4)
- 5) 72 °C 10 min
 - 6) 10 °C infinite

Agarose electrophoresis (90 V, 111 mA, 40 min):



➔ Result of PCR Colony: 1 clone of each was good

Precultures

3 mL LB medium + positive clones for each gene (PhaC2, PhaC3, PCT2)

Incubation 37 °C with shaking (around 16 h incubation)

➔ New transformation with the rest of ligation mix

Transformation (*E. coli* DH5-alpha with pSB1A3 ligated with gene) by heat shock

Thaw DH5-alpha competent bacteria in ice

100 µL of bacteria + 10 µL ligation mix

Incubation 20 minutes in ice

45 s at 42 °C

3 min in ice

Adding 900 µl of LB

Incubation 1 h, 37 °C

Spread on LB Ampicillin plates

Incubation overnight 37 °C

14 August 2016

➔ No colony for PCT2 transformation!

Miniprep from precultures

Transformed bacteria were stored at -80 °C in 50 % glycerol (2 aliquots for each preculture)

GenElute Plasmid Miniprep Kit (Sigma, PLN350-1KT)

Incubation 1 min for elution of DNA before centrifugation

pSB1A3-PhaC2 144 ng/µL

pSB1A3-PhaC3 96.1 ng/µL

pSB1A3-PCT2 143.3 ng/µL

Digestion

Mix:

	LDH1 gBlock gene (10 ng/µL)	pSB1A3 (X+P)
DNA	20 µL (200 ng)	50 µL
10X Buffer NEB CutSmart	3 µL	6 µL
enzyme XbaI	1 µL	2 µL
enzyme SpeI	1 µL	-
H2O (qs 30 µL)	5 µL	2 µL (qs 60 µL)

Incubation 1 h 37 °C

Dephosphorylation

Add 1 μ L alkaline phosphatase (FastAP #EF0654 Thermo Scientific) to the digestion mix (for LDH1 digestion or vector pSB1A3 digestion)

Incubation 30 min 37 °C

Inactivation of the enzyme: incubation 75 °C, 10 min

Recombinant vector digestion

Mix:

	pSB1A3-PhaC2	pSB1A3-PhaC3	pSB1A3-PCT2
DNA (around 200 ng or 600 ng)	2 μ L	2.5 μ L	6 μ L (around 600 ng)
Buffer NEB CutSmart 10X	3 μ L (2.1)	3 μ L (2.1)	3 μ L (CutSmart)
Enzyme 1	1 μ L (EcoRI)	1 μ L (EcoRI)	1 μ L (EcoRI)
Enzyme 2	1 μ L (SpeI)	1 μ L (SpeI)	1 μ L (XbaI)
H2O (qs 20 μ L)	14 μ L	13.5 μ L	10 μ L

Incubation 1 h 37 °C

DNA purification (LDH gene)

DNA clean & concentrator-5 kit (#D4003S Zymo Research)

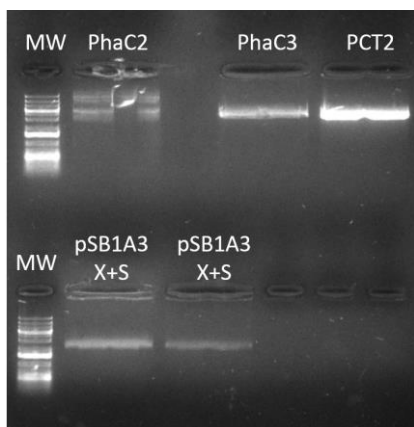
Ratio 5:1 of DNA binding buffer for LDH gene fragment

Elution 30 μ L water with incubation 3 min before centrifugation

LDH1 \rightarrow 6.9 ng/ μ L

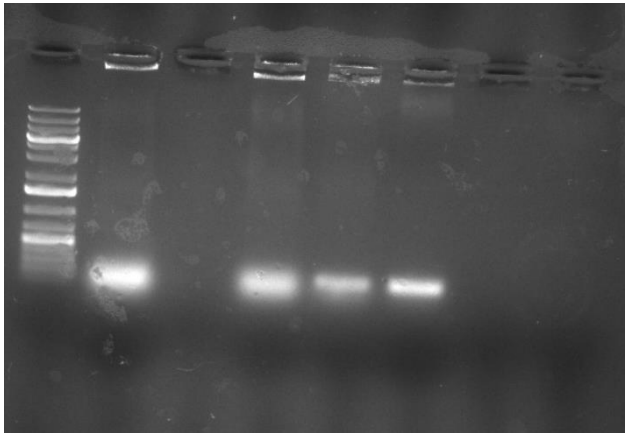
Agarose gel for digested recombinant vectors

All the digested samples were charged on the gel.



- \rightarrow Digestion was a failure for the 2 plasmids containing the PhaC2 and PhaC3 genes. The vector containing PCT2 is open but we have no way to be sure that the digestion was efficient for the 2 enzymes or just for one of them.

Colony PCR on the clones obtained after the 2nd transformation of the ligation mixes:



➔ PCR on colony failed

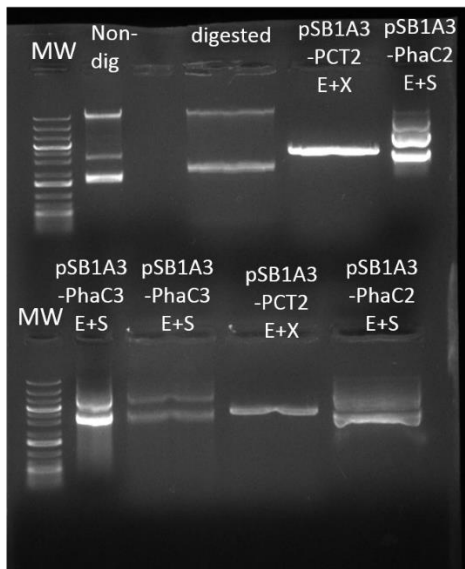
16 August 2016

Digestions

	LDH1 gBlock gene (10 ng/ μ L)	pSB1A3 (352 ng/ μ L)
DNA	30 μ L (300 ng)	2 μ L
10X Fastdigest buffer (ThermoScientific)	4 μ L (clear buffer)	2 μ L (green buffer)
enzyme XbaI	1 μ L	1 μ L
enzyme SpeI	1 μ L	1 μ L
H2O (qs 40 μ L)	4 μ L	14 μ L (qs 20 μ L)

	pSB1A3-PhaC2	pSB1A3-PhaC3	pSB1A3-PCT2
DNA (around 200 ng or 600 ng)	2 μ L	2.5 μ L	6 μ L (around 600 ng)
Buffer Fastdigest green 10X (ThermoScientific)	2 μ L	2 μ L	2 μ L
Enzyme 1	1 μ L (EcoRI)	1 μ L (EcoRI)	1 μ L (EcoRI)
Enzyme 2	1 μ L (SpeI)	1 μ L (SpeI)	1 μ L (XbaI)
H2O (qs 20 μ L)	14 μ L	13.5 μ L	10 μ L

Agarose gel 1 %



DNA purification (digested by X + S LDH1 gene)

DNA clean & concentrator-5 kit (#D4003S Zymo Research)

Ratio 5:1 of DNA binding buffer for LDH gene fragment

17 August 2016

- Autoclave
- Purification agarose gel via le kit « Zymoclean Gel DNA Recovery »
 - weight of the empty tube: 1 g
 - weight of the tube + vector PSB1A3 (X+S) n°1: 1,1 g is 100 mg
n°2: 1,1 is 100 mg
 - vector size: 2155 bp
 - Step 1: For 100 mg agarose gel, add 300 µL of ADB
 - Step 2: Put samples at 55 °C for 10 minutes and centrifuge at 300 rpm
 - Step 3: Transfer in the column
 - Step 4: Centrifugation while 1 minute at 13 000 rpm then suppression of the flow-through
 - Step 5: Add 200 µL of DNA Wash Buffer and centrifugation while 30 seconds X2
 - Step 6: Centrifugation empty for 2 minutes
 - Step 7: Add 10 µL DNA Elution Buffer, wait 2 minutes
 - Step 8: Centrifuge 1 minutes at 13 000 rpm
- Nano drop
 - OD vector pSB1A3 n°1: 39 ng/µL
 - OD vector pSB1A3 n°2: 43.2 ng/µL

18 August 2016

Chloramphenicol stock

50 ng/mL chloramphenicol stock (2.5 g in 50 mL ethanol 100 %)

30 aliquots stored at -20 °C

pSB1C3-mRFP

Solubilisation of pSB1C3-mRFP (2016 IGEM plate 5 - 1F) with 10 µL water nuclease-free (ThermoScientific)

Incubation 5 min RT

Transformation in *E. coli* DH5-alpha strain

Transformation

Thaw 25 µL DH5-alpha competent bacteria in ice

Add 2 µL of resolubilized pSB1C3-mRFP DNA from 2016 IGEM plate 5

Incubation 20 minutes on ice

1 min at 42 °C

3 min in ice

Adding 120 µL of LB

Incubation 1 h, 37 °C with shaking

Spread on LB Chloramphenicol plates

Incubation overnight 37 °C

Solubilisation of the primers iG051, iG052, iG049 and iG050 and aliquots of 10 times dilutions.

19 August 2016

Precultures

3 mL LB medium + 1.8 µL Chloramphenicol (50 mg/mL) → plates at 30 µg/mL chloramphenicol

- ➔ No conclusive results for PCR on colonies for pSB1C3-mRFP plates so we did precultures with 4 other clones (A, B, C and D)
- ➔ The Mastermix Taq DNA polymerase seems to not work anymore (after freezer problem, the polymerases lost their activities)

Q5 PCR for changing antibiotic resistance in pSEVA 224 plasmid

10 µL reaction buffer

1 µL dNTP mix

1 µL 10 times diluted primers (X2)

0.5 µL DNA template

0.5 µL Q5 DNA polymerase

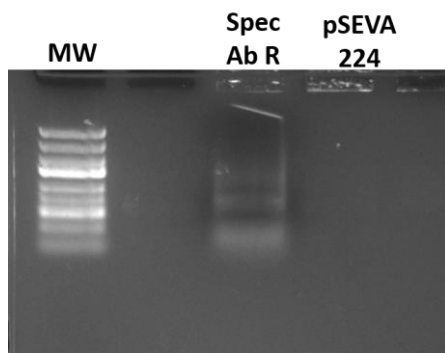
H2O qs 50 μ L (= 36 μ L)

2 different reactions:

- 1) Amplification of spectinomycin resistance gene with pCDF vector as DNA template (length expected = 1220 bp) primers used: iG050 and iG052 (1 min of DNA polymerization and primers annealing at 60 °C)
- 2) Amplification of pSEVA224 backbone without kanamycin resistance gene (pSEVA224 as DNA template; length expected = 4253 bp) primers used: iG049 and iG051 (3 min of DNA polymerization and primers annealing at 60 °C)

PCR program:

- 1) 95 °C 5 min
- 2) 95 °C 30 s
- 3) 60 °C 30 s
- 4) 72 °C 1 min or 3 min
35 repeats of the steps bloc from 2) to 4)
- 5) 72 °C 5 min
- 6) 10 °C infinite



➔ Amplification of pSEVA 224 failed ➔ why? Good primers so why!?

20 August 2016

DNA miniprep of pSB1C3-mRFP (clone A, B, C and D)

Stock in 50 % glycerol stored at -80 °C

GenElute Plasmid Miniprep Kit (Sigma, PLN350-1KT)

Incubation 2 min for DNA elution before centrifugation and centrifugation of 1 min30 s instead of 1 min.

Optional Wash Buffer step performed and use of nuclease-free water for elution.

Clone A: 240.3 ng/ μ L

Clone B: 150.3 ng/ μ L

Clone C: 111.0 ng/ μ L

Clone D: 76.6 ng/ μ L

PCR on DNA miniprep:

Primers iG001 and iG002.

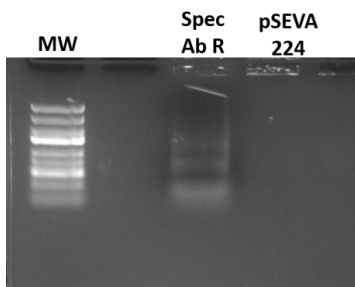
20 μ L mix per sample with:

- 10 μ L Green Taq Master Mix 2X (#K1081 Thermo Scientific)
- 0.25 μ L Forward primer iG001 (10 μ M)
- 0.25 μ L Forward primer iG002 (10 μ M)
- 9.5 μ L H₂O (qs 20 μ L)

PCR program:

- 7) 95 °C 5 min
- 8) 95 °C 30 s
- 9) 50 °C 30 s
- 10) 72 °C 1 min
30 repeats of the steps bloc from 2) to 4)
- 11) 72 °C 5 min
- 12) 10 °C infinite

Agarose gel 1 % for changing antibiotic PCR



➔ The amplification of pSEVA ➔ new attempt

Digestion vectors pSB1C3-mRFP (on DNA miniprep)

- 10 μ L plasmid DNA
- 3 μ L Buffer 10X green FD (FastDigest)
- 1 μ L EcoRI
- 1 μ L SpeI
- 15 μ L H₂O (qs 30 μ L)

Size expected: 2051 bp

Digestion was checked on agarose gel and the band corresponding to pSB1C3 digested by E+S was cut in order to recover the DNA.

22 August 2016

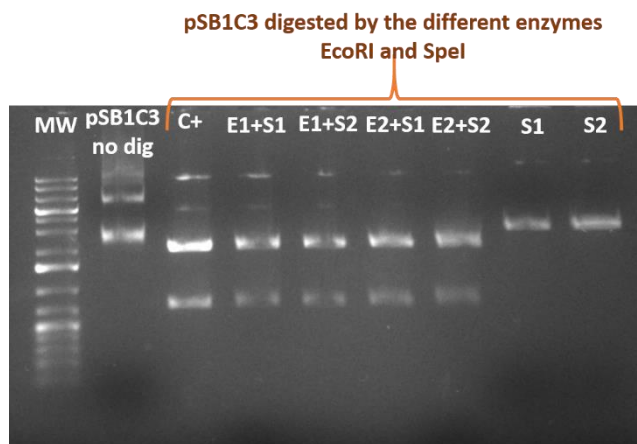
Digestion efficiency test of restriction enzymes EcoRI and SpeI

Mix:

- 4 μ L vector pSB1C3 (clone A)
- 3 μ L buffer 10X CutSmart or FastDigest Green
- 1 μ L EcoRI and/or SpeI
- 21 μ L or 22 μ L H₂O

Incubation 1 h 37 °C

Agarose gel 1 %



DNA extraction from agarose gel (linear vector extracted from clones A, B and C)

Zymoclean gel DNA Recovery kit #D4001S

Vector A: 20.9 ng/ μ L

Vector B: 13.3 ng/ μ L

Vector C: 13.1 ng/ μ L

Expected sizes after digestion by E+S:

pSB1C3 2051 bp

mRFP 742 bp

23 August 2016

· Digestion gBlocks by E+S

- 20 µL gBlocks DNA
- 3 µL 10X NEB Buffer (Cutmart)
- 1 µL EcoRI
- 1 µL SpeI
- 5 µL H₂O (qs 30 µL)

· Incubation 1 h at 37 °C

· Purification Agarose Gel with the kit « Zymoclean Gel DNA Recovery kit » (#D4001S Zymo Research)

- Cf protocol 17 August 2016

- Elution with 20 µL H₂O

· Nanodrop 1

- LDH2: 10.3 ng/µL
- Phac3: 5.9 ng/µL
- Phac 4: 8.5 ng/µL
- Pct 2: 8.9 ng/µL

too low concentration for ligation so concentration of the sample by evaporation of the solvent: incubation in Speed Vac device. Protocole « Vacuum Drive »

resuspend with 10 µL H₂O

· Nanodrop 2

- LDH2 = 15 ng/µL Ratio 260/280: 1.92
- Phac 3 = 8.8 ng/µL Ratio 260/280: 2.20
- PhaC4 = 9.2 ng/µL Ratio 260/280: 1.05
- Pct2 = 10.8 ng/µL Ratio 260/280: 1.88

Calculations:

genes	Longueur of genes	Amount (ng)	Concentration (ng/μL)	Volume (μL)
Plasmid psB1C3	2051 bp		20.9	2.4
PhaC3	1717 bp	209.3	8.8	23.8
LDH2	1227 bp	149.6	15	10
Phac 4	1720 bp	209.7	9.2	22.8
Pct 2	1612 bp	196.5	10.8	18.2

Calculation of amount of insert: $50 \times 5 \times \text{longueur gBlock} / \text{Longueur plasmid}$

Calculation of volume of insert: $\text{amount of insert} / \text{concentration of insert}$

As the sample was suspended in 10 μL, quantities not sufficient.

For Ligation Mix:

Insert	DNA (μL)	Vector (μL)	Buffer T4 Ligase 10X (μL)	T4 Ligase (μL)	H2O (qs 20 μL)
Pct2	9	0.96	2	0.5	7.54
LDH2	9	1.29	2	0.5	7.21
Phac4	9	0.96	2	0.5	7.54
Phac3	9	0.96	2	0.5	7.54

- Incubation at 22 °C of the ligation Mix during 10 min
- 5 μL of DNA for the transformation of 50 μL chemically competent cells

Transformation

- Use protocol of Bacterial transformation by heat shock
 - > Thaw competent cells on ice
 - > mixed 50 μL of competent bacteria with 5 μL of DNA
 - > incubate on ice for 20 minutes

- > placing the tubes at 42 °C for 1 min then put 3 min in ice
- > Add 250 µL of LB and incubate 1 h at 37 °C
- > Spread on box LB Chloramphenicol (30 µg/mL)
- > incubation overnight 37 °C

- Poured 6 plates of 25 mL of LB agar + chloramphenicol (30 µg/µL)
- Incubation at 37 °C

24 August 2016

- Digestion gBlocks by E+S
 - 20 µL gBlocks DNA
 - 3 µL 10X NEB Buffer (Cutmart)
 - 1 µL EcoRI
 - 1 µL SpeI
 - 5 µL H₂O (qs 30 µL)
- Incubation 1 h at 37 °C
- Purification Agarose Gel with the kit « Zymoclean Gel DNA Recovery kit » (#D4001S Zymo Research)

Elution with 20 µL nuclease-free water

PhaC3 (E+S) → 12.2 ng/µL

PhaC4 (E+S) → 6.3 ng/µL

LDH2 (E+S) → 16.8 ng/µL

PCT2 (E+S) → 12.4 ng/µL

Concentration of the DNA: around 1 h in evaporator

DNA resuspension with 11 µL water

- ➔ First concentration failed so second concentration of DNA performed in evaporator (1 h)

25 August 2016

Resuspension of DNA with 11 µL water

PhaC3 (E+S) → 10.4 ng/µL

PhaC4 (E+S) → 10.6 ng/µL

LDH2 (E+S) → 27.0 ng/μL

PCT2 (E+S) → 20.1 ng/μL

➔ DNA concentration by water evaporation failed!

Ligation genes inside pSB1C3 (ratio 5:1)

	PhaC4	PhaC3	PCT2	LDH2
Buffer T4 DNA ligase (10X)	2 μL	2 μL	2 μL	2 μL
T4 DNA ligase	0.5 μL	0.5 μL	0.5 μL	0.5 μL
Vector	0.96 μL	0.96 μL	0.96 μL	0.96 μL
Insert (gene)	7.9 μL	8.05 μL	3.91 μL	2.2 μL
H2O (qs 20 μL)	8.63 μL	8.49 μL	12.6 μL	14.3 μL

10 min incubation at 22 °C

Transformation

- Thaw 50 μL bacteria (*E. coli* DH5-alpha) chemo-competent
- Add 5 μL DNA (mix ligation)
- Incubation 20 min on ice
- Heat shock: 45 s at 42 °C
- Incubation 3 min on ice
- Add 950 μL LB
- Incubation 1 h at 37 °C with shaking
- Centrifugation 1 min at 4000 rpm
- Throw away supernatant
- Resuspension with the rest of supernatant (around 50 μL)
- Spread on plates LB Chloramphenicol (30 μg/mL)

26 August 2016

Preparation plates LBC (30 μg/μL)

Ligation (ratio 1:1)

	PhaC4	PCT2	PhaC3	LDH2
Buffer T4 DNA ligase (10X)	1 μL	1 μL	1 μL	1 μL
T4 DNA ligase	0.5 μL	0.5 μL	0.5 μL	0.5 μL
Vector	2.39 μL	2.39 μL	2.39 μL	2.39 μL
Insert (gene)	3.96 μL	1.95 μL	4.02 μL	1.11 μL
H2O (qs 10 μL)	2.15 μL	4.15 μL	2.08 μL	5.0 μL

Incubation 16 h 16 °C

27 August 2016

- PCR amplification of LDH_V2, PCT_V2, PHAC_V3 and PHAC_V4

Mix: Vf= 50 µL

For 5 reactions

5X Q5 Reaction Buffer	10 µL	50 µL
10 mM dNTP	1 µL	5 µL
10 µM Fw	2.5 µL	12.5 µL
10 µM Rv	2.5 µL	12.5 µL
DNA	1 µL	-
Q5 (0.2 U/µL)	0.5 µL	2.5 µL
H2O	32.5 µL	162.5 µL

- Solubilization of primers

For PR_IG063: Add 167 µL H2O for [100 µM] ➤ Aliquot 10 µL into 90 µL H2O [10 µM]

For PR_IG064: Add 246 µL H2O for [100 µM] → Aliquot 10 µL into 90 µL H2O [10 µM]

- Expected length:

Ldh_V2= 1248 bp $\sim \pm$ 2 nts

Pct_V2= 1633 bp

PhaC_V3= 1738 bp

PhaC_V4= 1692 bp

- Cycle:

98 °C	30 sec
98 °C	30 sec
55 °C	30 sec
72 °C	1 min
72 °C	2 min
10 °C	∞

↻ x35

28 August 2016

Transformation *E. coli* DH5-alpha

- Thaw 25 μ L chemo-competent bacteria
- Add 5 μ L DNA (mix ligation) (2 μ L of pSB1C3-mRFP from clone A as positive transformation control)
- Incubation 20 min on ice
- Heat shock: 45 s at 42 °C
- Incubation 3 min on ice
- Add 900 μ L LB
- Incubation 1 h20 at 37 °C with shaking
- Centrifugation 1 min 30 s at 5000 rpm
- Throw away all the supernatant
- Resuspension with 100 μ L LB medium
- Spread on plates LB Chloramphenicol (30 μ g/mL)
- Incubation overnight 37 °C

29 August 2016

The GFP gene contained in Gen4_BNR vector (provided by Cécile) was amplified by PCR with Q5 DNA polymerase with primers allowing to add the prefix and suffix regions at both sides of the amplified region.

Mix:

- 10 μ L Q5 reaction buffer 5X
- 1 μ L dNTP mix
- 2.5 μ L 10 μ M Forward Primer (iG063)
- 2.5 μ L 10 μ M Reverse Primer (iG064)
- 1 μ L DNA template
- 0.5 μ L Q5 DNA polymerase
- 32.5 μ L Nuclease-free water (qs 50 μ L)

PCR program:

- 1) 98 °C 30 s
- 2) 98 °C 30 s
- 3) 55 °C 30 s
- 4) 72 °C 1 min
35 repeats of the steps bloc from 2) to 4)
- 5) 72 °C 2 min
- 6) 10 °C infinite

➔ After checking on agarose 1 % gel, we determined that the GFP was successfully amplified.

Digestion

The amplified fragment was digested by EcoRI and PstI during 1 h at 37 °C. The reaction was performed in NEB CutSmart buffer.

The digested mix was purified by using the kit Wizard SV Gel and PCR Clean-Up System (Ref: A9282, Promega).

30 August 2016

Ligation

The GFP fragment was then ligated separately in pSEVA 224 or pSEVA 2311 digested by E+P in a ratio 5:1.

The ligation mix was stored at -20 °C until transformation.

1st September 2016

- Gel electrophoresis of the PCR amplification (27 October)

➡ Good length

- PCR Purification with a final volume = 50 µL
 - Nanodrop

Genes	Concentration (ng/µL)	Ratio 260/280
LDH_V2	109.5	1.82
PCT_V2	56.5	1.80
PHAC_V3	63.6	1.82
PHAC_V4	88.1	1.81

2 September 2016

- Digestion

Genes	Quantity of DNA (µL)	Restriction Enzymes (µL)	Buffer Digest NEB 2.1 (µL)	Water Qs 50 µL (µL)
LDH2 (109.5 ng/µL)	10	EcoR1 HF: 1 µL Pst1:1 µL	5	33
PCT2 (56.5 ng/µL)	20	EcoR1 HF: 1 µL Pst1:1 µL	5	23
PhaC3 (63.9 ng/µL)	17.5	EcoR1 HF: 1 µL Pst1:1 µL	5	25.5
PhaC4 (88.1 ng/µL)	12.5	EcoR1 HF: 1 µL Pst1:1 µL	5	30.5

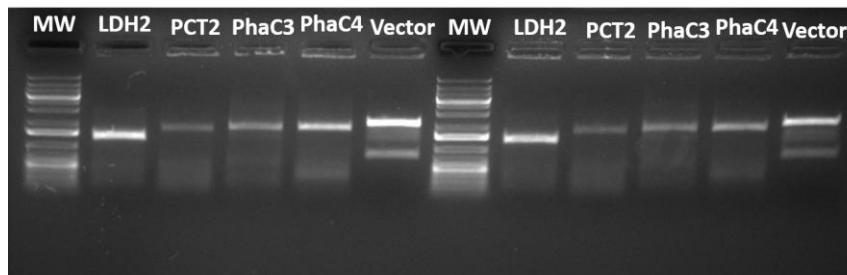
Vector PsB1C3 (240.3 ng/ μ L)	4.5	EcoR1 HF: 1 μ L Pst1:1 μ L	5	38.5
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Incubation 1 h at 37 °C and after incubation 20 min at 80 °C.

- Gel Extraction

50 mL of Agarose 1 % with 2.5 μ L of midorigreen.

Migration 25 min at 100 mv.



*Digestion mixes with EcoRI and PstI were loaded on the gel.

3 September 2016

DNA extraction from agarose gel

Kit: Wizard SV Gel and PCR Clean-Up System (Ref: A9282, Promega)

pSB1C3 → 6.4 ng/ μ L

LDH2 → 4.8 ng/ μ L

PCT2 → 2.9 ng/ μ L

PhaC3 → 4 ng/ μ L

PhaC4 → 5.8 ng/ μ L

DNA concentration by water evaporation:

pSB1C3 → 7.1 ng/ μ L

LDH2 → 6.5 ng/ μ L

PCT2 → 5.5 ng/ μ L

PhaC3 → 4.0 ng/ μ L

PhaC4 → 5.0 ng/ μ L

➔ DNA concentration still seems not efficient

DNA transformation

- Thaw 50 μ L chemo-competent DH5-alpha *E. coli*

- Add 5 μL DNA (mix ligation with promoters from pSEVA 2311 or 224)
- Incubation 25 min on ice
- Heat shock: 45 s at 42 °C
- Incubation 3 min on ice
- Add 250 μL LB
- Incubation 1 h 15 at 37 °C with shaking
- Spread on plates LB Kanamycin (50 $\mu\text{g/mL}$) (50 μL of bacteria on one plate and 10 μL on another one for each vector)
- Incubation overnight 37 °C

➔ The cells didn't express detectable fluorescence. A collaboration was setting up with Paris-Bettencourt IGEM team in order to characterize the IPTG inducible promoter by GFP expression. They also tried to characterize the cyclohexanone inducible promoter by the same way but it was a failure due to reaction between cyclohexanone and polystyrene (PS) from 96 wells plates.

4 September 2016

Ligation in pSB1C3

	LDH2 (6.5 ng/ μL)	PCT2 (6.5 ng/ μL)	PhaC3 (4.0 ng/ μL)	PhaC4 (5.0 ng/ μL)
Vector	4.2 μL	4.2 μL	4.2 μL	4.2 μL
Insert (gene)	2.8 μL	0.5 μL	0.5 μL	0.5 μL
Buffer T4 DNA ligase (10X)	2.39 μL	2.39 μL	2.39 μL	2.39 μL
T4 DNA ligase	3.96 μL	1.95 μL	4.02 μL	1.11 μL
H2O (qs 10 μL)	2.15 μL	4.15 μL	2.08 μL	5.0 μL

Incubation 1 h 16 °C

Poor plates LBC (30 $\mu\text{g/mL}$ chloramphenicol)

Transformation

- Thaw 25 μL chemo-competent DH5-alpha *E. coli*
- Add 4 μL DNA (mix ligation)
- Incubation 25 min on ice
- Heat shock: 45 s at 42 °C
- Incubation 3 min on ice
- Add 225 μL LB
- Incubation 1 h at 37 °C with shaking
- Spread 120 μL on plates LBC
- Incubation overnight 37 °C

7 September 2016

- Digestion of gene

Genes	Quantity of DNA (μL)	Restriction Enzymes (μL)	Buffer Digest NEB 2.1 (μL)	Water Qs 50 μL (μL)
LDH2 (109.5 ng/μL)	10	EcoR1 HF: 1 μL Pst1:1 μL	5	33
PCT2 (56.5 ng/μL)	20	EcoR1 HF: 1 μL Pst1:1 μL	5	23
PhaC3 (63.9 ng/μL)	17.5	EcoR1 HF: 1 μL Pst1:1 μL	5	25.5
PhaC4 (88.1 ng/μL)	12.5	EcoR1 HF: 1 μL Pst1:1 μL	5	30.5

Incubation 1 h at 37 °C and after incubation 20 min at 80 °C.

8 September 2016

- Digestion of backbone IGEM

Mix: Enzyme Master Mix E+P

- 0.5 μL NEB 2.1 Buffer
- 0.5 μL BSA (Bovine Serum Albumin)
- 0.5 μL EcoR1-HF
- 0.5 μL Pst1
- 0.5 μL Dnp1
- 18 μL H2O

Mix of 4 μL of linearized backbone and 4 μL of master mix enzyme

incubation of all the solution at 37 °C during 30 min and then, heat deactivation during 20 min at 80 °C.

- Ligation

Genes	length genes	Volume Final	Concentration Final	amount for 1 μL (Cf/Vf)
PSB1C3	2037 bp	8 μL	100 ng	25 ng = 2 μL
LDH_V2	1227 bp	50 μL	1095 ng	21.9 ng/μL
PCT_V2	1612 bp	50 μL	1130 ng	22.6 ng/μL
PhaC_V3	1717 bp	50 μL	1118.25 ng	22.365 ng/μL
PhacC_V4	1720 bp	50 μL	1101.25 ng	22.025 ng/μL

Amount calculation of insert for Ligation with NEBio Calculator:

Ratio 1 : 1		
Genes	Amount	Volume for deduced
LDH_V2	15.06 ng	0.7 µL
PCT_V2	19.78 ng	0.9 µL
PhaC_V3	21.07 ng	0.9 µL
PhaC_V4	21.11 ng	0.96 µL

Mix of Ligation:

- 2 µL of PSB1C3
- Volume calculate of digested insert
- 1 µL T4 Ligase Buffer
- 0.5 µL T4 Ligase
- Qs 10 µL

incubation mix at 16°C during 30 min and after heat kill for 20 min at 80 °C

- Transformation by heat shock

With 2 µL of Ligation mix and 25 µL of Competent Cells.

- 2 µL of Ligation with 25 µL of competent cells.
- Put the mix 20 min in the ice.
- Put the mix at 42 °C while 45 seconds
- 2 min on the ice.
- Add 950 µL of LB
- Recovery 1 h at 37 °C with agitation
- 7 000 rpm during 1 min
- Suppression of the flow-throw
- Resuspend with the remaining
- Spread on plates (put the simple in the middle of the plates)
- Incubation overnight 37 °C

- Plates preparation

1 plates = 25 mL of LB Agar + 15 µL of chloramphenicol [30 µg/mL]

$$Vi = \frac{30 \mu g \times 25 mL}{50\,000 \mu g} = 15 \mu g$$

Preculture

3 mL LB liquid medium + 1 µL *E. coli* DH5-alpha bacteria from stock at -80 °C

9 September 2016

- Preparation of chemo-competent *E. coli* DH5-alpha by heat shock
- WORK STERILE
- Launch a pre-culture of the strain in LB at 37 ° C
- Inoculate 25 mL of LB 1/50 (500µl) with the pre-culture
- Let it growth at 37 ° C with stirring until OD_{600 nm} = 0.5-0.7
- After 1 h30, OD= 0.9
- For Spectrometer measurements, 1 mL of LB for the blank
- A dilution was performed to obtain 0.5 OD_{600 nm} and the culture was split into 2 falcons with 27.5 mL of culture
- Cells are cooled 10 min on ice
- Centrifugate culture 6 min at 4000 rpm at 4 °C
- Suppression of the flow-through
- Dilution of the pellet in 1/2 volume of cold CaCl₂ 0.1 M (13.75 mL)
- Cells cooled on ice during 20 min
- Culture centrifugated 6 min at 4000 rpm at 4 °C
- Suppression of the flow-through
- Resuspension the pellet in 1 / 50e of volume (550 µL) of cold CaCl₂ 0.1 M + 10 % glycerol
- $$Vi = \frac{\frac{1}{100} \times 550}{5/100} = 110 \mu L$$
- The mix is 440 µL CaCl₂+ 110 µL glycerol 50 %
- 22 aliquots of 50 µL bacteria

11 September 2016

- PCR Amplification of LDH2, PCT2, PHAC3, PHAC4 with Q5 DNA polymerase

For 50 µL reaction

- 25 µL of Q5 Master Mix
- 2.5 µL of Forward Primer (IG063)
- 2.5 µL of Reverse Primer (IG064)
- 0.5 of Template DNA
- 19.5 of H₂O

Cycle:

Temperature	Time
98 °C	30 seconds
98 °C	30 seconds
55 °C	30 seconds
72 °C	1 minute
72 °C	2 minutes
10 °C	Pause

↑
x35
↓

- PCR Colony LDH2 with One Taq

For 25 µL to 1 Reaction → Vf = 125 µL

Mix:

- 25 µL of One Taq Standard Reaction Buffer
- dNTPs= 2.5 µL
- 2.5 µL of Forward Primer (IG001)
- 2.5 µL of Reverse Primer (IG002)
- 0.625 µL of One Taq DNA Polymerase
- 91.9 µL of Nuclease Free Water

Cycle:

Temperature	Time
94 °C	30 seconds
94 °C	30 seconds
50 °C	1 minute
68 °C	1 min 45 s
68 °C	5 minutes
10 °C	Pause

↑
x30
↓

12 September 2016

- MiniPrep Colonies LDH
 - 3 µL of LB
 - Take one of the colony in the plates
 - Incubated at 37 °C overnight
 -
- Digestion Backbone IGEM pSB1C3 linearized by E+P

Mix: Enzyme Master Mix E+P for 22 reaction

- 2 µL NEB 2.1 Buffer
- 2 µL BSA (Bovine Serum Albumin)
- 2 µL EcoRI-HF
- 2 µL PstI
- 2 µL DnpI
- 72 µL H₂O

16 µL of linearized backbone and 16 µL of the MasterMix enzyme

Incubation at 37 °C during 30 min

Enzyme inactivation with 20 min incubation at 80 °C.

Precultures

5 mL LB medium

+ 5 µL antibiotic (Kanamycin 50 mg/mL for pSEVA 212S, Spectinomycin 50 mg/mL for pSEVA 424 and Gentamycin 10 mg/mL for pSEVA 628S)

+ some bacteria (sent as bacteria aliquots in LB medium by de Lorenzo)

Incubation 37 °C overnight

13 September 2016

Miniprep of pSEVA plasmids

2 aliquots stocks in 50 % glycerol stored at -80 °C

GenElute Plasmid Miniprep Kit (Sigma, PLN350-1KT)

3 DNA vectors: pSEVA 424 (equivalent to the pSEVA 224 but with spectinomycin resistance gene instead of kanamycin, pSEVA 212S and pSEVA 628S used for genes deletion in genomic DNA in *Pseudomonas putida*)

Digestion of the “old” gene by E+P

Ligation mix:

- 2 µL of PSB1C3
- Volume calculate of digested insert
- 1 µL T4 Ligase Buffer
- 0.5 µL T4 Ligase
- Qs 10 µL

Incubation 16°C 30 min and heat kill for 20 min at 80 °C

Ratio 1 : 5		
Genes	Amount	Volume for deduced
LDH_V2	75.29 ng	3.44 μ L
PCT_V2	98.92 ng	4.38 μ L
PhaC_V3	105.4 ng	4.71 μ L
PhaC_V4	105.5 ng	4.79 μ L

Ratio 1 : 2		
Genes	Amount	Volume for deduced
LDH_V2	30.12 ng	1.37 μ L
PCT_V2	39.57 ng	1.71 μ L
PhaC_V3	42.15 ng	1.88 μ L
PhaC_V4	42.22 ng	1.92 μ L

- Minipreps DNA Purification System kit “Wizard Plus”

- Take 2 μ L of overnight culture
- Centrifuge at top speed for 1 min
- Suppression of the flow-through
- Resuspend pellet with 250 μ L of Cell Resuspension Solution
- Add 250 μ L of Cell Lysis Solution and invert 4 times to mix
- Add 10 μ L of Alkaline Protease Solution and invert 4 time to mix
- Incubate 5 min at room temperature
- Add 350 μ L of Neutralization Solution and invert 4 times to mix
- Centrifuge at top speed for 10 min at room temperature
- Insert Spin Column into Collection Tube
- Decant cleared lysate into Spin Column
- Centrifuge at top speed for 1 min
- Discard flow-through
- Add 750 μ L of Wash Solution and centrifuge at top speed 1 min
- Discard flow-through
- Repeat with 250 μ L of Wash Solution
- Centrifuge at top speed for 2 min
- Transfer Spin Column to 1.5 mL tube
- Add 100 μ L of Nuclease free water to the Spin Column
- Centrifuge at the top speed for 1 min
- Discard column

- Purification of Amplification PCR LDH2, PCT2, PHAC3, PHAC4
- Nanodrop

For the gene:

Genes	Concentration (ng/μL)	Ratio 260/280
LDH2	62.7	1.78
PCT2	52.8	1.79
PHAC3	50.3	1.76
PHAC4	69.7	1.78

For the LDH colonies:

Colonies	Concentration (ng/μL)	Ratio 260/280
LDH_n°1	70	1.92
LDH_n°2	31	2.06

- Digestion “New” genes LDH2, PCT2, PHAC3, PHAC4

Digestion by EcoR1-HF+Pst1

Genes	Quantity of DNA (μL)	Restriction Enzymes	Buffer Digest NEB 2.1 (μL)	H2O (μL)
LDH2 (62.7 ng/μL)	16	1 μL EcoR1-HF 1 μL Pst1	5	27
PCT2 (52.8 ng/μL)	19	1 μL EcoR1-HF 1 μL Pst1	5	24
PHAC3 (63.9 ng/μL)	16	1 μL EcoR1-HF 1 μL Pst1	5	27
PhAC4 (69.7 ng/μL)	14.5	1 μL EcoR1-HF 1 μL Pst1	5	28.5
pSB1C3 (240.3 ng/μL)	4.5	1 μL EcoR1-HF 1 μL Pst1	5	38.5

Other Digestions:

Genes	Quantity of DNA (μL)	Restriction Enzymes	Buffer Digest NEB 2.1 (μL)	H2O (μL)
PHAC3	16	1 μL Xba1 1 μL Pst1	5	27
PHAC4	14.5	1 μL Xba1 1 μL Pst1	5	28.5
PCT2	19	1 μL EcoR1-HF 1 μL Spe1	5	24

Incubation at 37 °C during 30 min and then, enzymes deactivation by heat kill for 20 min at 80 °C.

- PCR Purification
 - Nanodrop Vf = 50 µL

Genes	Concentration (ng/µL)	Ratio 260/280
LDH_V2	62.7	1.78
PCT_V2	52.8	1.79
PHAC_V3	50.3	1.76
PHAC_V4	69.7	1.78

14 September 2016

The promoter region of the vectors pSEVA 224 and pSEVA 2311 were amplified by PCR with Q5 DNA polymerase with primers allowing to add the prefix and suffix regions at both sides of the amplified region.

Mix:

- 10 µL Q5 reaction buffer 5X
- 1 µL dNTP mix
- 2.5 µL 10 µM Forward Primer (iG063)
- 2.5 µL 10 µM Reverse Primer (iG064)
- 1 µL DNA template
- 0.5 µL Q5 DNA polymerase
- 32.5 µL Nuclease-free water (qs 50 µL)

PCR program:

- 1) 98 °C 30 s
 - 2) 98 °C 30 s
 - 3) 55 °C 30 s
 - 4) 72 °C 1 min
- 35 repeats of the steps bloc from 2) to 4)
- 5) 72 °C 2 min
 - 6) 10 °C infinite

➔ After checking on agarose 1 % gel, we determined that the fragments were successfully amplified.

Digestion

The amplified fragments were digested by EcoRI and PstI during 1 h at 37 °C. The reaction was performed in NEB CutSmart buffer.

The digested mixes were purified by using the kit Wizard SV Gel and PCR Clean-Up System (Ref: A9282, Promega).

15 September 2016

Ligation

The 2 fragments were then ligated separately in linear pSB1C3 provided by IGEN Headquarters in a ratio 5:1.

The ligation mix was used for transformation in *E. coli* DH5-alpha.

Transformation

- Thaw 25 µL chemo-competent DH5-alpha *E. coli*
- Add 3 µL DNA (mix ligation)
- Incubation 20 min on ice
- Heat shock: 45 s at 42 °C
- Incubation 3 min on ice
- Add 225 µL of LB medium
- Incubation 1 h at 37 °C with shaking
- Spread 120 µL on plates LBC
- Incubation overnight 37 °C

Preculture of clones in 3 mL LB Cam. From the ligation.

16 September 2016

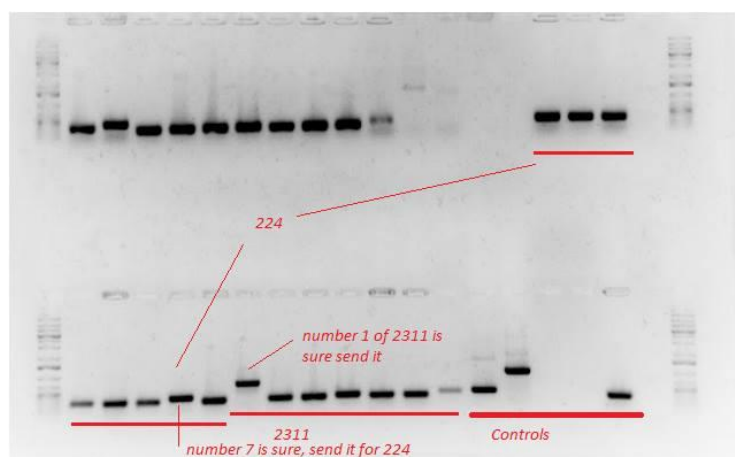
Colonies PCR

Mix (25 µL total volume reaction):

- 12.5 µL DreamTaq PCR Mastermix 2X (ThermoScientific)
- 2.5 µL 10X DreamTaq Green Buffer (ThermoScientific)
- 0.25 µL Forward Primer (0.5 µM) iG001
- 0.25 µL Reverse Primer (0.5 µM) iG002
- 9.5 µL H2O filtrated (qs 25 µL)

+ clones

Agarose gel 1 %



Precultures

Positive clones selected from the agarose gel results were picked and used to inoculate 3 mL of LBC (25 µg/mL chloramphenicol) medium.

Observation

➔ All the precultures have grown

- PCR colony

Mix:

- Dream Taq MM 2X	10 µL	280 µL
- Fw	1.5 µL	42 µL
- Rv	1.5 µL	42 µL
- DNA	1 µL	-
- H2O	6 µL	168

X28

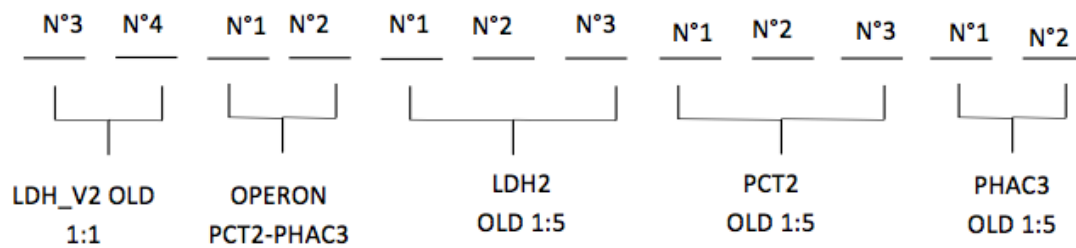
Just to remind:

Position E5: Mix without clone

Position E6: Mix with pLAC into pSB1C3

Position E7: Mix with mRF-HisTag into Psb1C3

Migration map:



➤ Expected Bands

LDH_V2= ~1550

PCT_V2=~1950

PHAC_V3=~2050

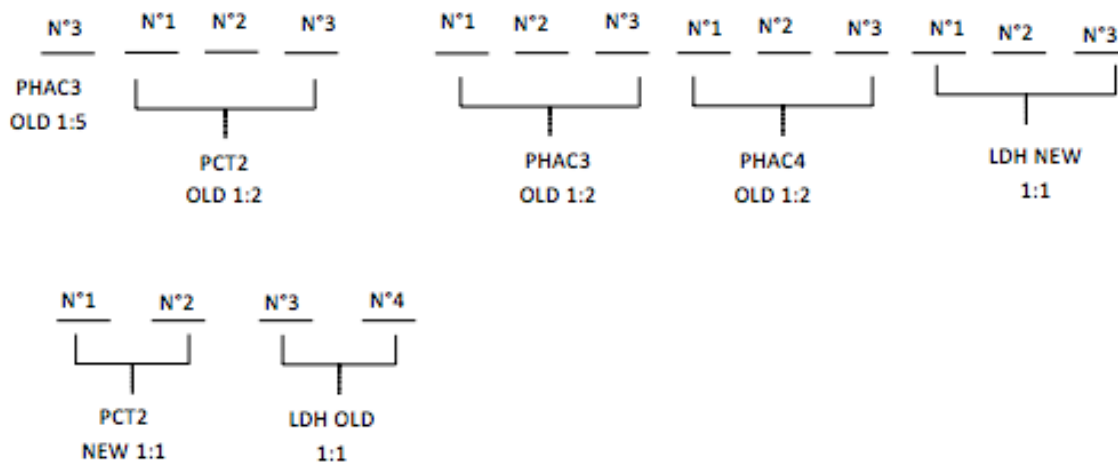
PHAC_V4=~2000

➤ Cycle

95 °C	3 min
95 °C	30 sec
50 °C	30 sec
72 °C	1.15 min
72 °C	5 min
10 °C	∞

X30

Gel Plan migration:



20 September 2016

Precultures

3 mL LB medium in 15 mL Falcon tube+ 1 μ L *P. putida* KT2440 from -80 °C stock

Incubation 30 °C overnight with shaking.

- PCR Gibson of pct_V2, phac_V3, phac_V4, pSB1B3 HQ, pSB1C36 mRFP-HisTag

Mix:

Vf= 50 μ L

- 5X Q5 Reaction Buffer
- 10 mM dNTP
- 10 μ M Fw
- 10 μ M Rv
- DNA
- Q5 (0.2 U/ μ L)
- H2O

10 μ L	70 μ L
1 μ L	7 μ L
2.5 μ L	- μ L
2.5 μ L	- μ L
1 μ L	-
0.5 μ L	3.5 μ L
32.5 μ L	227 μ L

→
x7

- Solubilization of primers

For PR_IG075: Add 209 μ L H2O for [100 μ M]

For PR_IG076: Add 198 μ L H2O for [100 μ M]

For PR_IG077: Add 242 μ L H2O for [100 μ M]

For PR_IG078: Add 236 μ L H2O for [100 μ M]

For PR_IG079: Add 257 μ L H2O for [100 μ M]

For PR_IG080: Add 300 μ L H2O for [100 μ M]

For PR_IG081: Add 235 μ L H2O for [100 μ M]

For PR_IG082: Add 229 μ L H2O for [100 μ M]

For PR_IG083: Add 238 μ L H2O for [100 μ M]

For PR_IG084: Add 266 μ L H2O for [100 μ M]

Aliquot 1/10 of each primer

- Gel purification of PCT2, PhaCv3, PhaCv4
- Purification

21 September 2016

- Gel Extraction pSB1C3 + mRFP-Histag

Gel weight= 300 mg

- PCR clean-up pct2, phac_v3, phac_v4

Nanodrop:

Genes	Concentration (ng/ μ L)	Ratio 260/280
PCT_V2	152	1.82
PHAC_V3	130.7	1.84
PHAC_V4	132.9	1.83
pSB1C3 IHQ	108.3	1.80
pSB1C3 Biobrick	14.7	1.74

- Gibson
 - Ratio Calculation

➔ Pct2-phac3 into pSB1C3

Mix:

pSB1C3 IGHQ	0.92 μ L
H2O	6.75 μ L
PCT2	1.04 μ L
PHAC3	1.29 μ L

➔ Pct2-phac4 into pSB1C3 IGHQ

Mix:

pSB1C3 IGHQ	0.92 μ L
H2O	6.76 μ L
PCT2	1.04 μ L
PHAC4	1.27 μ L

➔ Pct2-phac3 into pSB1C3 Biobrick

Mix:

pSB1C3 Biobrick	6.80 μ L
H2O	0.87 μ L
PCT2	1.04 μ L
PHAC3	1.29 μ L

➔ Pct2-phac4 into pSB1C3 Biobrick

Mix:

pSB1C3 Biobrick	6.80 μ L
H2O	0.89 μ L
PCT2	1.04 μ L
PHAC4	1.27 μ L

Add 10 μ L of HIFI DNA Assembly MM in each.

And 10 μ L of hifi with 10 μ L of positive control

Keep on ice!

➤ Incubation: 50 °C, 15 minutes then on ice

- Chemically Competent Cells Transformation
 - ➔ Add 2 μ L of the chilled assembled to 25 μ L competent cells
 - ➔ Waiting 30 min in ice
 - ➔ Heat shock at 42 °C for 30 sec
 - ➔ Transfer the tube on ice for 2 min
 - ➔ Add 950 μ L LB for each
 - ➔ Incubation: 60 min, 37 °C, 250 rpm
 - ➔ Spread 100 μ L on the plate
 - ➔ Incubation 37 °C overnight

23 September 2016

- Gibson plate observation
 - ➔ DH5-alpha pct2-phac3-pSB1C3 iGHQ 14 clones
 - ➔ DH5-alpha pct2-phac4-pSB1C3 iGHQ 13 clones
 - ➔ DH5-alpha pct2-phac3-pSB1C3 Biobrick 1 clones
 - ➔ DH5-alpha pct2-phac4-pSB1C3 Biobrick 5 clones

- PCR Colony

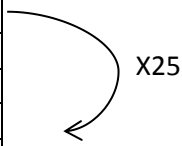
Mix:

- DreamTaq Master Mix 2X	10 μ L	350 μ L
- PR_IG001	1.5 μ L	52.5 μ L
- PR_IG002	1.5 μ L	52.5 μ L
- DNA	1 μ L	-
- H2O	6 μ L	210 μ L

→
X35

Cycle:

95 °C	3 min
95 °C	30 sec
50 °C	30 sec
72 °C	1.30 min
72 °C	2 min
10 °C	∞



x25

- Result Gibson

N°2 pct2 phac4 pSB1C3 iGEM HQ

N°1 pct2 phac4

N°5 pct2 phac4 pSB1C3 Biobrick

- Preculture in LB+ Cam (10 µg/mL)
 - Miniprep of the cultures
 - Gel Verification of Gibson PCR
- ➔ Don't work

Growth experiments

Stock in 50 % glycerol stored at -80 °C

Experiments performed in triplicates on 96 wells plate with LB medium as blank.

The plate was incubated at 30 °C with shaking during 20 h after inoculating with 1/1000 dilution of preculture.

The OD at 600 nm was read every 20 s.

The experiments were repeated 3 consecutive days from fresh precultures started the day before.

The results were sent to Imperial College iGEM team for collaboration along with the results of carbon source tests performed in July.

27 September 2016

- Sequencing
- ➔ WatchBox 1887819 (27.09.16)

28th September 2016

- Gibson
- ➔ Calculation for Ratio 1:1

Mix DNA 1:

pct2	0.62 µL
Phac3	0.76 µL
H2O	8.62 µL

Mix DNA 2:

pct2	0.66 µL
Phac3	0.81 µL
H2O	8.63 µL

Mix DNA3:

pct2	0.62 µL
Phac4	0.75 µL
H2O	8.63 µL

Mix DNA 4:

pct2	0.66 µL
Phac4	0.80 µL
H2O	8.54 µL

- ➔ Calculation for Ratio 1:2

Mix 5:

Diluted pct2	1.04 µL
Diluted Phac3	1.29 µL
H2O	6.75 µL
IGHQ Vector	0.92 µL

- ➔ Mix 6:

Diluted pct2	1.04 µL
Diluted Phac4	1.27 µL
H2O	6.76 µL
IGHQ Vector	0.92 µL

→ Mix 7:

Diluted pct2	1.04 µL
Diluted Phac3	1.29 µL
H2O	4.27 µL
Biobrick Vector	3.40 µL

→ Mix 8:

Diluted pct2	1.04 µL
Diluted Phac4	1.27 µL
H2O	4.29 µL
Biobrick Vector	3.40 µL

→ Put 15 min to 50 °C and after at -20 °C

29th September 2016

- Transformation
 - 2 µL product + 25 µL of Dh5-alpha cells+ 950 µL SOC Medium
 - 1 h Recovery
- Culture plate LB Cam (10 µg/mL)
- Preparing plate

$$Ci = 50 \text{ mg/mL} \quad vi = \frac{10 \text{ µg} \cdot 25 \text{ mL}}{50000} = 5 \text{ µl}$$

$$Cf = 10 \text{ µg/mL}$$

$$Vf = 25 \text{ mL}$$

- Purification PCR of gBlock fragment
- Nanodrop
 - Nothing ☹

30th September 2016

- Concentration / SpeedVac
- Verification Gel of purifications
- PCR amplification gBlock

4 October 2016

Gibson assembly 2nd step

pSB1C3 IGEM Headquarters 110.3 ng/μL

pSB1C3 mRFP 14.1 ng/μL

Mix Gibson from the 1st step giving the operon:

- 1) PCT2+PhaC3 65.5 ng/μL
- 2) PCT2+PhaC4 60.8 ng/μL
- 3) PCT2+PhaC3 63.4 ng/μL
- 4) PCT2+PhaC4 65.7 ng/μL

Gibson ratio 1:1

Vector amplified from pSB1C3-mFRP	1)	Vector	3.40 μL (50 ng)
		Insert (mix 1)	1.21 μL
		H2O (qs 10 μL)	5.40 μL
	2)	Vector	3.40 μL (50 ng)
		Insert (mix 2)	1.30 μL
		H2O (qs 10 μL)	5.30 μL
	3)	Vector	3.40 μL (50 ng)
		Insert (mix 3)	1.25 μL
		H2O (qs 10 μL)	5.35 μL
	4)	Vector	3.40 μL (50 ng)
		Insert (mix 4)	1.20 μL
		H2O (qs 10 μL)	5.40 μL
Linear vector pSB1C3 provided by IGEM Headquarters	5)	Vector	0.91 μL (100 ng)
		Insert (mix 1)	2.42 μL
		H2O (qs 10 μL)	6.68 μL
	6)	Vector	0.91 μL (100 ng)
		Insert (mix 2)	2.60 μL
		H2O (qs 10 μL)	6.49 μL
	7)	Vector	0.91 μL (100 ng)
		Insert (mix 3)	2.50 μL
		H2O (qs 10 μL)	6.60 μL
	8)	Vector	0.91 μL (100 ng)
		Insert (mix 4)	2.41 μL
		H2O (qs 10 μL)	6.68 μL

Add 10 μL NEB HiFi DNA Assembly Mastermix 2X

Incubation 50 °C, 15 min

Cool on ice

Transformation

- Thaw 50 μ L chemo-competent DH5-alpha *E. coli*
- Add 2 μ L DNA (mix Gibson)
- Incubation 30 min on ice
- Heat shock: 30 s at 42 °C
- Incubation 2 min on ice
- Add 950 μ L NEB medium (#B9020S)
- Incubation 1 h at 37 °C with shaking
- Centrifugation 7000 rpm 1 min
- Resuspend cells with rest of supernatant after throwing away the most of it
- Spread all the content on plates LBC
- Incubation overnight 37 °C

7 October 2016

Solubilisation of IDT gBlocks in 100 μ L of nuclease-free water and short centrifugation

Incubation 50 °C during 20 min

Vortexing and then, short centrifugation again

-PCR of gBlock genes

Mix:

- 10 μ L Q5 reaction buffer 5X
- 1 μ L dNTP mix
- 2.5 μ L 10 μ M Forward Primer (iG063)
- 2.5 μ L 10 μ M Reverse Primer (iG064)
- 1 μ L DNA template
- 0.5 μ L Q5 DNA polymerase
- 32.5 μ L Nuclease-free water (qs 50 μ L)

Mix for 5 reactions

PCR program:

- 1) 98 °C 30 s
- 2) 98 °C 30 s
- 3) 55 °C 30 s
- 4) 72 °C 1 min
35 repeats of the steps bloc from 2) to 4)
- 5) 72 °C 2 min
- 6) 10 °C infinite

Colony PCR on Gibson transformation

- 12.5 µL DreamTaq Green PCR Master Mix 2X (Thermo Scientific, #K1081)
- 1.25 µL 10 µM Forward Primer (iG001)
- 1.25 µL 10 µM Reverse Primer (iG002)
- 10 µL Nuclease-free water (qs 25 µL)

+ clone

Mix for 23 reactions

PCR program:

- 1) 95 °C 5 min
- 2) 95 °C 30 s
- 3) 50 °C 30 s
- 4) 72 °C 3 min
30 repeats of the steps bloc from 2) to 4)
- 5) 72 °C 10 min
- 6) 10 °C infinite

-Nanodrop of PCR genes purification of 3.09.16

Genes	Concentration (ng/µL)	Ratio 260/280
LDH_V2	138.2	1.88
PCT_V2	115.6	1.92
PHAC_V3	125.4	1.91
PHAC_V4	134.7	1.91

8 October 2016

➔ Problem with several PCR reaction tubes ➔ we do them again

Colony PCR on Gibson transformation

- 12.5 µL DreamTaq Green PCR Master Mix 2X (Thermo Scientific, #K1081)
- 1.25 µL 10 µM Forward Primer (iG001)
- 1.25 µL 10 µM Reverse Primer (iG002)
- 10 µL Nuclease-free water (qs 25 µL)

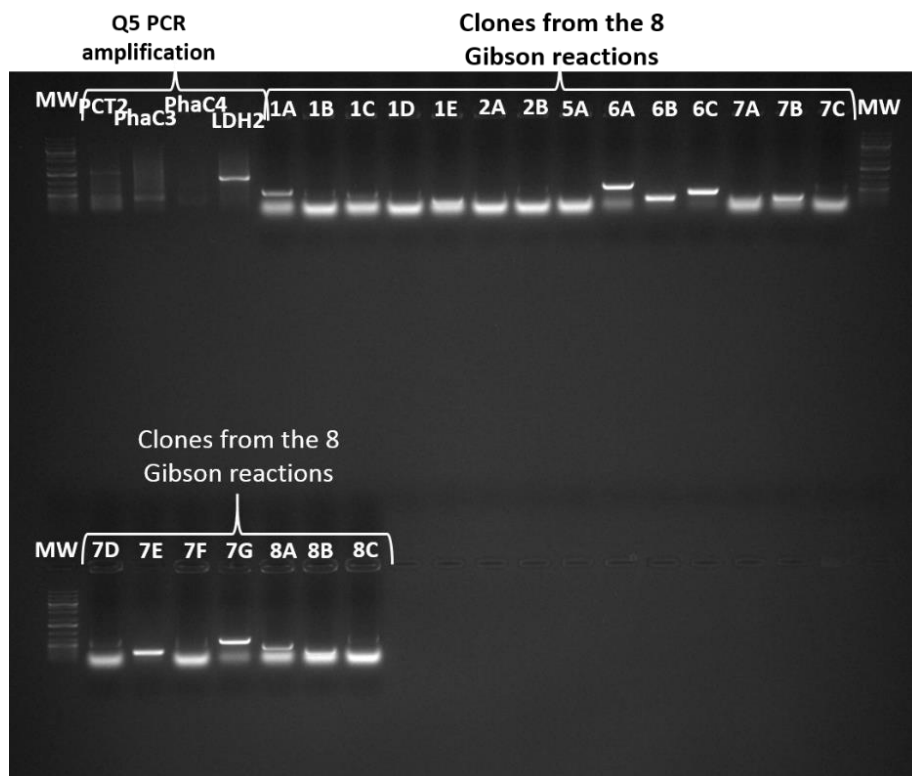
+ clone

Mix for 9 reactions

PCR program:

- 1) 95 °C 5 min
- 2) 95 °C 30 s
- 3) 50 °C 30 s
- 4) 72 °C 3 min
30 repeats of the steps bloc from 2) to 4)
- 5) 72 °C 10 min
- 6) 10 °C infinite

Agarose gel 1 %



Digestion E+P

Mix: Vf= 100 µL

DNA	50 µL
Buffer 2.1	10 µL
EcoR1	1.5 µL
Pst1	1.5 µL
H2O	37 µL

PCR clean-up

➤ Nanodrop

7 October		
Genes	Concentration (ng/μL)	Ratio 260/280
LDH_V2	37.7	2.04
PCT_V2	29.8	2.07
Phac_V3	35.7	2.04
Phac_V4	40.3	2.05

30 September		
Genes	Concentration (ng/μL)	Ratio 260/280
LDH_V2	8.8	2.49
PCT_V2	7.7	2.32
Phac_V3	11.0	2.68
Phac_V4	8.1	2.46
21 September		
Genes	Concentration (ng/μL)	Ratio 260/280
LDH_V2	7.9	2.54
PCT_V2	7.8	2.59
Phac_V3	7.0	2.35
Phac_V4	7.3	2.31

3 September		
Genes	Concentration (ng/μL)	Ratio 260/280
LDH_V2	16.3	2.20
PCT_V2	7.1	2.79
Phac_V3	8.2	2.30
Phac_V4	9.0	2.52

9 October 2016

Pouring of 46 LBC (10 μg/mL chloramphenicol) plates + 5 LBK (50 μg/mL kanamycin) plates + 2 LBS (50 μg/mL spectinomycin) plates

10 October 2016

Concentration measurements:

LDH 7.10: 37.7 ng/μL

PCT 7.10: 29.8 ng/μL

PhaC3 7.10: 35.7 ng/μL

PhaC4 7.10: 40.3 ng/μL

LDH 3.09: 16.3 ng/ μ L
PCT 3.09: 7.1 ng/ μ L
PhaC3 3.09: 8.2 ng/ μ L
PhaC4 3.09: 9.0 ng/ μ L

LDH 21.09: 7.9 ng/ μ L
PCT 21.09: 7.8 ng/ μ L
PhaC3 21.09: 7.0 ng/ μ L
PhaC4 21.09: 7.3 ng/ μ L

LDH 30.08: 8.8 ng/ μ L
PCT 30.08: 7.7 ng/ μ L
PhaC3 30.08: 11.0 ng/ μ L
PhaC4 30.08: 8.1 ng/ μ L

11 October 2016

- Preparation 1L water+ 0.1 % HCOOH
- Test of solubility -> 10 μ L of standard in 90 μ L Solvent

➤ Anthocyanin

- ➔ Isobutanol = not soluble
- ➔ DMSO = not soluble
- ➔ Ethyl Acetate = not soluble
- ➔ CH₃CN 60 % = not soluble
- ➔ CH₃CN 100 % = not soluble
- ➔ ETOH = not soluble
- ➔ HCL = ~soluble
- ➔ Methanol + Water + HCL = ~soluble
- ➔ Methanol+ HCL= ~soluble

➤ Quercetin

- ➔ EtOH = soluble

➤ Maldivin

- ➔ Soluble in Methanol HCL

Bettencourt Collaboration = we decided to do spectrometry masse instead of HPLC because it is more fast in time and the quercetin will be passed in negative mode but the maldivin and anthocyanin will be passed in negative mode.

12 October 2016

- Solution Stock
➔ At 2 mg/mL in EtOH 100 %

2 mg -> 1 mL EtOH

12.3 mg -> 6.15 mL EtOH

- Standard range in H₂O mQ

- Quercetin

Isotopic mass = 302.042664 Da

Formula = C₁₅H₁₀O₇

Solution Stock: [2000 mg -> 1000 mL]

- [20 mg/1000 mL]
➔ 6 µL in 594 µL H₂O mQ -> Solution 1
- [10 mg/L]
➔ 300 µL of 1 + 300 µL H₂O -> Solution 2
- [5 mg/L]
➔ 300 µL of 2 + 300 µL H₂O -> Solution 3
- [1 mg/L]
➔ 100 µL of 3 + 400 µL H₂O -> Solution 4
- [0.5 mg/L]
➔ 250 µL of 4 + 250 µL H₂O -> Solution 5
- [0.1 mg/L]
➔ 50 µL of 5 + 200 µL H₂O -> Solution 6

- Maldivin

Isotopic mass = 330.073955 Da

Formula = C₁₇H₁₅O₇

Solution Stock: 50 μL + 450 μL MeOH-HCl 2 M \rightarrow [100 mg/L]

- [20 mg/1000 mL]
 \rightarrow 100 μL in 400 μL H₂O mQ or CH₃CN 60 % \rightarrow Solution 1
- [10 mg/L]
 \rightarrow 250 μL of 1 + 250 μL H₂O mQ or CH₃CN 60 % \rightarrow Solution 2
- [5 mg/L]
 \rightarrow 250 μL of 2 + 250 μL H₂O mQ or CH₃CN 60 % \rightarrow Solution 3
- [1 mg/L]
 \rightarrow 100 μL of 3 + 400 μL H₂O mQ or CH₃CN 60 % \rightarrow Solution 4
- [0.5 mg/L]
 \rightarrow 250 μL of 4 + 250 μL H₂O mQ or CH₃CN 60 % \rightarrow Solution 5
- [0.1 mg/L]
 \rightarrow 50 μL of 5 + 200 μL H₂O mQ or CH₃CN 60 % \rightarrow Solution 6
- Plaque MS
 \rightarrow 75 μL of each dilution + 75 μL Solution C

Solution C = 95 % acetate + 5 % carbonate ammonium 10 mM

- Vial
 \rightarrow 200 μL of each dilution in insert into vial for test HPLC

13th October 2016

- Ligation 1:10 with different $V_f = 25 \mu\text{L}$ or $V_f = 20 \mu\text{L}$

Length

- GB3 = 2038 bp
- IGHQ = 2037 bp

DNA Mix:

7 October 2016

LDH + IGFQ (100 ng)	
Vector	0.92 μ L
H2O	2.92 μ L
LDH	16.2 μ L

LDH + GB3 (50 ng)	
Vector	2.16 μ L
H2O	9.77 μ L
LDH	8.08 μ L

PCT + IGHQ (75 ng)	
Vector	0.69 μ L
H2O	0.78 μ L
PCT	20.09 μ L

PCT + GB3(50 ng)	
Vector	2.16 μ L
H2O	4.46 μ L
PCT	13.39 μ L

PHAC3 + IGHQ (75 ng)	
Vector	0.69 μ L
H2O	1.45 μ L
PHAC3	17.85 μ L

PHAC3 + GB3(50 ng)	
Vector	2.16 μ L
H2O	5.95 μ L
PHAC3	11.90 μ L

PHAC4 + IGHQ (75 ng)	
Vector	0.92 μ L
H2O	2.05 μ L
PHAC4	21.12 μ L

3rd September 2016

LDH + GB3 (50 ng) 1:10	
Vector	2.16 μ L
H2O	0.89 μ L
LDH	18.68 μ L

PHAC3 + GB3 (75 ng) 1:5	
Vector	2.16 μ L
H2O	2.51 μ L
PHAC3	30.33 μ L

21th September 2016

PHAC4 + GB3(50 ng)	
Vector	2.16 μ L
H2O	7.29 μ L
PHAC4	10.56 μ L

30th September 2016

PHAC3 + GB3 (75 ng) 1:5	
Vector	2.16 μ L
H ₂ O	2.44 μ L
PHAC3	25.9 μ L

- Preparation of standard range for MS
20 mg/L; 10 mg/L; 5 mg/L; 1 mg/L
- Preparation of samples
75 μ L of supernatant of each + 75 μ L of solution C.

14 October 2016

- Transformation of the ligation product by heat shock
3 μ L of ligation product into DH5-alpha strain.
Recovery 1 hour.
spread on LB + chloramphenicol plates (25 μ g/ μ L).

15 October 2016

Digestion PhaC3_only and PCT2_only

- 30 μ L IDT gBlock gene
- 5 μ L buffer NEB CutSmart 10X
- 1 μ L EcoRI
- 1 μ L SpeI
- 13 μ L nuclease-free water (qs 50 μ L)

Incubation 1 h 37 °C

DNA clean & concentrator-5 kit (Zymo Research, D4003S)

DNA binding buffer added with 5:1 ratio

1 min incubation after adding 20 μ L of nuclease-free water before centrifugation for elution

Ligation

	Quick ligase	T4 DNA ligase
pSB1C3 digested by E+S (from clone B → 13.3 ng/μL)	6 μL	6 μL
gene digested by E+S	3 μL	3 μL
Buffer	10 μL Quick ligase buffer 2X (NEB)	2 μL T4 DNA ligase buffer 10X (NEB)
ligase	1 μL Quick ligase (NEB)	1 μL T4 DNA ligase
H2O (nuclease-free water)	-	8 μL

Transformation ligation mixes in pSB1C3

- Thaw 50 μL chemo-competent DH5-alpha *E. coli*
- Add 2 μL DNA (mix ligation)
- Incubation 20 min on ice
- Heat shock: 45 s at 42 °C
- Incubation 3 min on ice
- Add 450 μL LB medium
- Incubation 1 h at 37 °C with shaking
- Centrifugation 7000 rpm 3 min
- Throw away supernatant
- Resuspend cells with rest of supernatant (around 50 μL)
- Spread all the content on plates LBC (10 μg/mL chloramphenicol)
- Incubation overnight 37 °C

Transformation ligation mixes in pSEVA 224, 424 or 2311

- Thaw 50 μL chemo-competent DH5-alpha *E. coli*
- Add 2 μL DNA (mix ligation)
- Incubation 20 min on ice
- Heat shock: 45 s at 42 °C
- Incubation 3 min on ice
- Add 450 μL LB medium
- Incubation 1 h at 37 °C with shaking
- Centrifugation 7000 rpm 3 min
- Throw away supernatant
- Resuspend cells with rest of supernatant (around 50 μL)

- Spread all the content on plates LBK (50 µg/mL kanamycin) for pSEVA 224 or 2311 or LBS (50 µg/mL spectinomycin) for pSEVA 424
- Incubation overnight 37 °C

DNA PCR clean-up kit (NEB) for gBlock genes amplified by PCR

16 October 2016

➔ Our strain of DH5-alpha bacteria seems already resistant to spectinomycin ➔ test by preculture in 3 mL LBS (50 µg/mL spectinomycin) liquid medium

Concentration of amplified gBlocks after PCR clean-up

PhaC4: 86.6 ng/µL
 PhaC3: 120.6 ng/µL
 LDH2: 202.5 ng/µL
 PCT2: 154.6 ng/µL

Digestion by E+P of amplified genes

	PhaC3	PhaC4	PCT2	LDH2
DNA genes	7 µL	7 µL	5 µL	4 µL
Buffer NEB CutSmart	5 µL	5 µL	5 µL	5 µL
EcoRI	1 µL	1 µL	1 µL	1 µL
PstI	1 µL	1 µL	1 µL	1 µL
H2O nuclease-free	36 µL	36 µL	38 µL	39 µL

DNA clean & concentrator-5 kit (#D4003S Zymo Research)

Ratio 5:1 of DNA binding buffer

Incubation 1 min with 20 µL of nuclease-free water before centrifugation for elution

Precultures

2 mL LBC (25 µg/mL chloramphenicol) + clones from transformation of DH5-alpha *E. coli* with ligation mixes of pSB1C3 with PhaC3_only or PCT2_only

PCT_only: 9 clones from ligation with T4 DNA ligase and 5 clones from ligation with Quick ligase

PhaC3_only: 1 clone from Quick ligase and 2 clones from T4 DNA ligase

Incubation of the 17 precultures overnight at 37 °C with shaking.

17 October 2016

Miniprep

GenElute Plasmid Miniprep Kit (Sigma, PLN350-1KT)

3 clones for PhaC3 and 14 clones for PCT2 (see above)

Testing methods to detect the interest compound in HPLC

Samples run

- Preparation of samples
 - Vortex and samples centrifugation, 2 min, max speed.
 - Take 120 µL of supernatant of each and put them in each 1.5 tube.
 - Add 120 µL acetonitrile 100 %.
 - Centrifugation, 1 min, max speed.
 - Load 200 µL in the vials for HPLC.

18 October 2016

Colony PCR on the last trials of insertion of PhaC3, PhaC4, PCT2, LDH2, PhaC3_only and PCT2_only inside pSB1C3 and of insertion of PhaC4, PCT2 and LDH2 inside pSEVA224 and pSEVA2311 in process.

PCR Colonies:

20 µL mix per sample with:

- 10 µL Green Taq Master Mix 2X (#K1081 Thermo Scientific)
- 0.25 µL Forward primer iG001 (10 µM)
- 0.25 µL Forward primer iG002 (10 µM)
- 9.5 µL H2O (qs 20 µL)

+clone

PCR program:

- 7) 95 °C 5 min
- 8) 95 °C 30 s
- 9) 50 °C 30 s
- 10) 72 °C 2 min
30 repeats of the steps bloc from 2) to 4)
- 11) 72 °C 10 min
- 12) 10 °C infinite

HPLC Analysis (see Bettancout collaboration)

Sequencings of promoters isolated from 224 and 2311 plasmids and born by pSB1C3 came back positive.

20 October 2016

Gel electrophoresis with mixture from PCR on colonies obtained from PhaC3_only and PCT2_only ligations in pSB1C3.



*the two samples chosen are indicated by an arrow

Results for the sequencing of the promoters from pSEVA224 and pSEVA2311 came positives.

21 October 2016

Samples (promoters from pSEVA224 and pSEVA2311 and genes PhaC3_only and PCT2_only) sent to iGEM registry and to sequencing for PhaC3_only and PCT2_only.

24 October 2016

Sequencing results for PhaC3_oly and PCT2_only came positives.